SOME STUDIES ON *THEILERIA ANNULATA* WITH SPECIAL REFERENCE TO AN ATTENUATED VACCINE

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SUMMARY

The disease, tropical theileriosis, caused by the protozoan parasite Theileria annulata is assuming greater importance in many tropical and sub-tropical countries because of the improvement in the breeds and productivity of cattle in these countries. Methods of control of the disease are inadequate and a suitable means of immunization would be of great value to the respective livestock industries. The literature dealing with these aspects of the disease relevant to its importance and methods of control is reviewed. Methods of immunization are considered and work is described on the isolation, cultivation, attenuation and use of strains of the parasite. Special attention is paid to the medium used for growing the infected lymphoid cells and the factors contributing to optimum, satisfactory growth. The research described is concentrated on the preparation of vaccines attenuated in tissue culture. Details of the methods of adaptation to and attenuation in, tissue culture of strains of T.annulata, and experiments leading to the development, preservation and use of a definitive vaccine are described. The initial assessment of possible vaccinal value is based on the clinical and haematological response of susceptible bovine animals and the definitive vaccine used is an attenuated strain, initially isolated in Iran. This strain is investigated in respect of its safety in use, its properties as a field vaccine, its ability to withstand deep frozen

storage and its immunogenic characteristics. It is shown that the vaccine can be stored at -70°C. very satisfactorily using glycerol or dimethyl sulphoxide as cryoprotectants, that it is effective when administered subcutaneously and that it causes a readily detectable reaction in susceptible cattle, producing schizonts but not erythrocytic forms; and that it engenders immunity to a heterologous strain of the parasite. Experiments are described in which an immunity is shown to be produced in cattle which is strong at 4 months when challenged by a local heterologous strain and still exists, but at a lower level, 12 months after vaccination. Attenuation of T.annulata strains in tissue culture is discussed and a regimen for vaccination of calves and particular care that should be taken for vaccination of adult cattle are suggested. Some suggestions for future investigations are made.

CONTENTS

Chapter 1

INTRODUCTION

Theileria annulata infection which has been referred to as Egyptian fever, tropical piroplasmosis, tropical theileriosis and Mediterranean Coast fever is a tick-borne disease which is caused by the protozoan parasite T.annulata (Dschunkowsky and Luhs 1904). The causative agent is transmitted by ticks of the genus Hyalomma Koch, 1844. The disease occurs in Africa north of the Sahara, Southern Europe, the Middle East, Southern U.S.S.R. and India.

Cattle of European, Asiatic and African breeds, water buffalo (Bubalis bubalis Linn.) and the American bison (Bison bison Linn.) (Neitz 1957) are susceptible to the disease. Mortality in cattle depends on the susceptibility of the breed and the virulence of strains. Unlike East Coast fever, caused by Theileria parva, in which the mortality rate in adult grade cattle is over 80% according to most authors (Brocklesby, Barnett and Scott 1961; Wilde, Brown, Hulliger, Gall and MacLeod 1968), mortality due to T.annulata is much lower but can vary as for example in Palestine from 35 to 40% (Adler and Ellenbogen 1934) in Algeria from 20 to 40% (Donatien and Lestoquard 1938) and in India up to 76% (Sen and Srinivasan 1937).

Although the parasite has been recognised for a long time (Dschunkowsky and Luhs 1904) it was not until European pure-bred cattle were introduced into enzootic regions for improvement of the local breeds that the importance of the

disease was realised. It was desirable that the low productive indigenous breeds in enzootic regions should be replaced by breeds of higher productivity to cope with the increased consumption of livestock products arising as a result of improvement in social standards in developing countries. To bring this about, European breeds of cattle were introduced and programmes of upgrading of local stock instituted. The high susceptibility to T.annulata infection of these newly introduced breeds led to considerable losses from tropical theileriosis. MacHatti (1935) from his studies in Iraq concluded that at least 50% of cross-bred calves died of theileriosis each year. Yousif (1969) reported that of 100 European cows imported to Iraq and stationed in a modern government dairy farm, 44 became infected with theileriosis and 29 died of the disease. This was in spite of the practice of strict tick control measures. The reports published by the Veterinary Department of Iraq (1963, 1964 and 1965) show that 761, 6,912 and 9,111 cases of theileriosis, respectively, were recorded annually. Theileria annulata infection can, therefore, be classified as an important animal disease in those areas where it occurs.

Investigations into specific chemotherapy of the disease have so far achieved little success (Neitz 1957). In recent years the introduction of the new chlorinated and organophosphorus compounds as acaricides with residual effect has contributed considerably to the control of the tick vectors of the disease. The effects of these

acaricides are, however, reduced by the limitations of application and general methods of husbandry. Without rigorous control of the movement of stock, the acaricides, no matter how effective, cannot eliminate the disease. Supplementary measures are demanded and these could include elimination of carrier animals and artificial immunization. The elimination of the carrier animals in the enzootic area is very costly and uneconomical. The use of a blood vaccine was considered to be an effective method of controlling T.annulata infection (Sergent, Donatien, Parrot and Lestoquard 1945), but this method of vaccination seems to have fallen into disuse.

Some degree of success reported by various workers on the propagation of Theileria spp. in tissue culture and the application of T.annulata tissue cultured schizonts for vaccination (Tsur, Adler, Pipano and Senft 1964), encouraged the author to investigate the immunogenic properties of strains of T.annulata adapted to tissue culture and to attempt to standardize the method of vaccine production. A study of the reactions of animals to the resultant vaccine and of the duration of the immunity engendered was made.

Chapter 2

REVIEW OF THE LITERATURE

It is intended in this review of the literature on T.annulata and the disease which it causes to concentrate on those aspects of the subject most relevant to the present investigation. While mention will be made of other aspects, this will be brief only, as excellent reviews by Neitz (1957) and Barnett (1968) are available.

CLASSIFICATION

Classification of the family Theileriidae has always been in dispute. This has been, mainly, due to incomplete knowledge of the life cycles of the members of this family. Wenyon (1926) placed the genus Theileria (Bettencourt, Franca and Borges 1907) in the family Theileriidae and proposed the following classification.

Phylum	Protozoa
Class	Sporozoa
Subclass	Coccidiomorpha
Order	Coccidiida
Suborder	Piroplasmidea
Family	Theileriidae
Genus	Theileria

This classification has been subject to reviews and changes ever since its introduction. Hall (1953) proposed that genus Theileria be put in the order Babesiida of the subclass Haemosporidea. Neitz and Jansen (1956) reclassified the Theileriidae and maintained the genus Theileria with a single species T.parva while reviving

the previously used genus Gonderia (Du Toit 1918) and placed the rest of the members of the Theileriidae in this genus. The basis for their reclassification was the conception, of that time, that T.parva multiplied only by schizogony while multiplication of the members of the genus Gonderia was by schizogony as well as by binary fission of their erythrocytic forms. When Neitz (1962) showed that T.parva erythrocytic forms can divide and maintain themselves, this classification was abandoned. Levine (1961) because of the unknown parts of the life cycle proposed a new class "Piroplasmea" as an appendage to the phylum Protozoa, pending the clarification of the obscure points. The committee on taxonomy of the Society of Protozoologists (Honigberg, Balamuth, Bovee, Corliss, Gojdics, Hall, Kudo, Levine, Loeblich, Weiser and Wenrich 1964) decided that Piroplasms should be placed in a new sub-phylum Sarcomastigophora, super class Sarcodina, class Piroplasmea with a single order Piroplasmida. On the basis of this, Levine (1971) again reviewed the classification of Piroplasma and this time by taking into account the results obtained from electronmicroscope studies on the ultra structure of these micro-organisms presented the following classification.

Class	Piroplasmasida
Order	Piroplasmorida
Families	Babesiidae, Theileriidae, Dactylosomatiidae

T.annulata with synonyms, Piroplasma annulatum, Gonderia annulata, T.dispar, T.turkestanica and T.sergenti, is placed, in this classification, under the family Theileriidae. Four of the species named above have been proved to be synonyms of T.annulata but the fifth one, T.sergenti, according to Markov, Stepanov, Laptev, Duboyi and Storozhev (1964) on serological and xenodiagnostic bases is considered to be a good species. In the present author's view the classification adopted by Barnett (1968) is the simplest and most reasonable.

Class Piroplasmea

Order Piroplasmida

Families Theileriidae Babesiidae

Family Theileriidae:

Genera Theileria, Cytauxzoon, Haematoxenus

Genus Theileria:

1 - Theileria of cattle

a - Theileria mutans Group (T.mutans, T.orientalis)

b - Theileria sergenti

c - Theileria annulata

d - Theileria parva Group (T.parva, T.lawrencei "a modified strain of T.parva")

2 - Theileria of sheep and goats

a - Theileria ovis

b - Theileria hirci

3 - Theileria of other domestic ruminants (Examples; T.camelensis and T.tarandi-rangiferis)

4 - Theileria of other mammals

The validity of Theileria in 3 and 4 remains to be confirmed in the future as, so far, these parasites have only been found in the erythrocytes of peripheral blood and as they resemble Theileria they have been classified under this genus Theileria.

LIFE CYCLE

The life cycle of T.annulata, unlike that of T.parva has not been studied closely by many authors. Sergent, Donatien, Parrot and Lestoquard (1936a, 1936b, 1936c and 1945) have described the life cycle of T.dispar (= T.annulata) in the 2-host tick Hyalomma mauritanicum (= H.detritum Schulze 1919) and the bovine host. They are of the opinion that schizogony in the vertebrate host is followed by sporogony in the invertebrate host. According to these authors the erythrocytic forms of the parasite, when ingested by larvae, escape from the erythrocytes and congregate in masses in the intestine. The above authors stated that syngamy occurs within the intestine about 13 hours after tick attachment. Zygotes appear in the epithelial cells of the gut 7 hours later. They become encysted during the period of the larval moult. After the moult, the zygotes emerge from the cysts and develop into ookinetes. Ookinetes enter the body cavity and migrate to the salivary glands, where they penetrate the cells and turn into sporonts. Before and during the nymphal moult the sporonts develop into multinucleated masses, the sporoblasts. When the adult tick attaches

itself to a new susceptible host and commences to feed, the sporoblasts break up into numerous uninucleated sporozoites which enter the salivary ducts, and are injected into the new host. These authors are of the opinion that at least 60 hours are needed for this process of production of sporozoites from sporoblasts to take place. It is noteworthy to mention here that Mazlum (1969) claims to have transmitted the parasite by injecting, subcutaneously, crushed infected unfed H.dromedarii Koch, 1844 into susceptible animals, on 2 occasions. On the basis of his findings he concludes that the parasite, in tick transmission, passes to the host animal on the same day that ticks attach themselves to the host. The author of the present text (unpublished work) could not repeat these results when he used infected H.excavatum Koch, 1844 which is the main vector of T.annulata in the Middle East. It seems that more, carefully planned, experiments are needed for further elucidation of this stage of the life cycle. However, there are no other reports either to confirm or disprove the theory presented by Sergent et al. (1945) for the development of T.annulata in vector ticks. As a somewhat similar theory was suggested by Cowdry and Ham (1932) for T.parva and later was disputed by other investigators, one is hesitant to accept this theory without reserve.

Once the parasites are inoculated into the susceptible bovine host they probably migrate to the lymphatic tissue and after an incubation period which is on the average 14

days (Sergent et al. 1945) become patent and can be detected in the lymph node draining the site of tick attachment. At this stage the parasite is referred to as the macroschizont or agamont. One or 2 days later, a thermal rise indicates that the parasites have invaded the blood circulation. On this day or a day later macroschizonts can be found in other organs and lymph nodes. Gradually in addition to the macroschizont another form of the parasite appears which is referred to as the microschizont or gamont. The microschizonts unlike macroschizonts have numerous chromatin dots which are round, these are believed finally to break up into individual bodies that invade the red cells to become the erythrocytic forms. These 2 forms of the parasite, macroschizonts and microschizonts, are also referred to as Koch's blue bodies. In recovered animals the erythrocytic forms persist for a long period, in one case as long as 11 years (Sergent et al. 1945).

Although there are disagreements about the mode of the development of the parasite in the tick, there is general agreement that the erythrocytic forms are the final stage of the parasite. These forms of the parasite have to be ingested by the vector tick and, then they undergo a process of development, which is not very clearly known at the present time, before the parasite can be transmitted from one host to another. Recently Pipano (1972) claimed to be able to induce T.annulata infection in splenectomized calves by inoculating the

erythrocytic forms obtained from 2 donor calves which had recovered 167 and 432 days before, respectively. According to this author 6 out of 10 calves, which had been infected in this way, died of severe theileriosis. Even if the possibility of natural infection, by infected ticks, is excluded it is quite possible that the donors in his experiments still harboured schizonts. Sergent et al. (1945) described the detection of schizonts, in very small numbers, in the lymph node or other organs of apparently healthy cattle slaughtered at the abattoir. They also described the detection of schizonts in the lymph node of a calf, removed by operation, 40 days after its recovery from theileriosis. The author of this text in his previous work (unpublished) while making tissue culture from the spleen of a calf, 90 days after it had recovered from experimental theileriosis, was able to grow lymphoid cells containing T.annulata schizonts. If the experiments reported by Pipano (1972) can be repeated, excluding with certainty all the possibilities of passaging undetectable schizonts, then a new field for investigation is opened up. Splenectomy of the donor calves can be exploited, in this respect, in any future experiment. By splenectomizing the donor calf the number of the erythrocytic forms increases and the amount of blood needed to be inoculated into the recipient can be considerably reduced, thus the possibility of passaging any existing schizont will be diminished.

TRANSMISSION

A - Biological. Theileria annulata can be transmitted by ticks of the genus Hyalomma. Six species have been reported to transmit this protozoan (Neitz 1957). Attempts to transmit T.annulata with Rhipicephalus appendiculatus Neumann,1901 (Sergent, Donatien, Parrot, Lestoquard and Plantureux 1927a), R.bursa Canestrini and Fanzago 1877 (Sergent et al. 1945) and Anopheles maculipennis var. labranchiae (Sergent 1940) have failed. In each part of the world where the parasite occurs, one or other species of Hyalomma is the main vector. Ticks usually take up the parasite at the larval or the nymphal stage and transmit it at the nymphal or adult stage. Sergent, Donatien, Parrot and Lestoquard (1931) in their 26 experiments failed to transmit the parasite transovarially by H.mauritanicum but in all experiments they succeeded in transmitting the parasite from stage to stage. Kornienko and Shmyreva (1944) were the first authors to describe the hereditary transmission of T.annulata in H.turkmeniense (= H.excavatum). They had collected female ticks from cattle with T.annulata infection. The larvae and nymphae that resulted from these ticks were fed on rabbits and 50 of the adults were fed on a calf in the autumn. This calf did not become infected that autumn but it showed the infection next spring. One cannot be absolutely sure that this calf was not infected by ticks which are quite abundant in spring time. Their results were not confirmed by Delpy (1949) who from his observations concluded that

there was no evidence of hereditary transmission of T.annulata in H.detritum, H.excavatum, H.rufipes glabrum Delpy, 1949 and H.savignyi Gervais, 1844. Ray (1950) reported the transmission of T.annulata transovarially through 4 generations of H.savignyi. He stated that the parasite could be transmitted at the adult stage and not at the larval or the nymphal stage. As he was using calves to feed ticks in his experiments there is the possibility that he was simply transmitting T.annulata from one stage to another. In addition to the previous authors who did not accept the transovarial transmission of T.annulata Daubney and Said (1951) confirmed that there was no hereditary transmission by H.excavatum. It is certain that T.annulata can be transmitted from one stage to another but hereditary transmission is most unlikely to happen.

B - Artificial. Unlike T.parva that cannot easily be transmitted by blood inoculation, T.annulata can be so passaged from an infected animal, during the thermal reaction of the disease, to a clean susceptible host. Transmission is possible using blood, organ suspension or any body fluids containing macroschizonts. As will be seen later, transmission can be carried out using tissue cultured schizonts. Artificial transmission of theileriosis was described by Sergent et al. (1945). They studied 28 strains in a period of 22 years. Some of these strains were maintained in animals at the laboratory for long periods. One of the strains (Kouba) was

maintained for 12 years by carrying out 223 serial passages. The interesting point found, in the course of the serial passages, was the phenomenon that the parasite, when passaged serially from animal to animal, lost the ability to produce microschizonts and the erythrocytic forms. This phenomenon appeared in one of the strains (Kouba) after 18 consecutive passages and in another (St. Charles) after 11 passages. The authors mentioned that the calves inoculated with these strains did not show erythrocytic forms even after splenectomy and the ticks which were fed on these calves did not become infected. The present author (unpublished data) maintained a strain of T.annulata, Strain No. 3, by serial passages from animal to animal, for nearly 9 years. This strain also lost its ability to produce erythrocytic forms and propagated only by producing macroschizonts. This rather easy method of transmission has made comprehensive studies on virulence, antigenicity and immunogenicity of different strains of T.annulata possible. It has also contributed to the introduction of vaccines against tropical theileriosis.

C - Intra-uterine. This form of transmission of T.annulata was reported by Magneville (1925) and Sergent et al. (1945). The former author described the disease in a 7 days old calf out of a premune dam. The latter authors could not induce this form of transmission experimentally but in one case detected very few erythrocytic forms in the blood of a newly born calf from

a dam that was showing 75 parasites per 1,000 red cells. No schizonts could be found in this calf, either by microscopic examination of the smears from liver and spleen or the inoculation of the suspensions prepared from these organs. It is very likely that the case of theileriosis reported by Magneville (1925) had been induced by infected ticks after birth. However, the intra-uterine form of transmission, if taking place at all, must be of little significance.

SYMPTOMS

Symptoms vary according to the duration and severity of the disease. The degree of manifestation depends on the susceptibility of the animals and the virulence of the strains (Neitz 1957). After exposure to tick infestation, the incubation period varies from 8 to 30 days with an average of 14 days (Sergent et al. 1945). After inoculation of infected blood, the period varies from 10 to 30 days, the average being 17 days (Sergent et al. 1945). In rare cases incubation periods longer than 30 days, even up to 10 months, were observed in 11 out of 1,260 calves infected artificially by Sergent et al. (1945). Brumpt (1923 and 1924) observed incubation periods as long as 7 months. The present author (unpublished) has observed an incubation period of 6 months which was followed by a very severe manifestation of the disease and the animal died in 2 days.

The form that the disease takes, depending on the virulence of the strain and the susceptibility of the

animal, can be peracute, acute or subacute. The most common form is the acute form (Neitz 1957), which will be described here in detail. This form of the disease can be seen in both tick transmitted and artificially produced infection. A rise in body temperature to 104°F. or above, which may persist from 5 to 20 days, is the first clinical manifestation of the disease. Schizonts can be detected in lymph nodes, liver and spleen from the onset of fever until the end of pyrexia (Sergent et al. 1945). The erythrocytic forms usually appear 2 or 3 days after the appearance of schizonts. The present author (unpublished) has observed instances of theileriosis, artificially produced, in which the erythrocytic forms have appeared simultaneously with the schizonts and even preceeding the appearance of schizonts. The erythrocytic forms can infect 100% of the red cells. According to Sergent et al. (1945) in one case there were 5,000 of these forms of the parasite in 1,000 red cells. The animal shows inappetance, cessation of rumination, drooling from the mouth, serous nasal discharge, swelling of the superficial lymph nodes (Neitz 1957). Swelling of the eye lids or protrusion of the eye ball due to hyperplasia of the local lymphatic tissue may also be seen (Khalifa and Kadhim 1967). This has been supported by my own personal observations. Other symptoms are lachrymation, accelerated pulse, general weakness, great reduction in and even loss of milk production (Neitz 1957). Anaemia (Sergent et al. 1945) and bilirubinaemia and

bilirubinuria (Neitz 1957) are always present. The conjunctiva shows, in addition to anaemic features and in some cases icterus, petechiae which can be from one to a few mm. in diameter. Neitz (1957) mentioned that at the beginning of fever in this form of the disease, the faeces are firm but diarrhoea soon sets in. In the present author's experience which is supported by the findings of Sergent et al. (1945) constipation is a typical characteristic of acute T.annulata infection. In some cases diarrhoea may also be seen. Animals become markedly emaciated and lie down. At this stage fever may stop and the animals resume feeding and recover or may become comatose, hypothermic and die.

The peracute form is very similar to the above but has a much shorter course and generally terminates fatally (Neitz 1957). The subacute form is often encountered in animals infected with a relatively mild strain of T.annulata. The symptoms are less marked than in the acute form. The body temperature may fluctuate, sometimes, for as long as 15 days. Animals usually recover from this form of the disease.

PATHOLOGY

The lesions vary according to the duration and severity of the disease. Not many authors have attempted to describe the pathological changes due to T.annulata infection. Sergent et al. (1945) described the macroscopical lesions occurring in T.annulata infection in North Africa. Neitz (1957) in his review also has

given a detailed description of gross pathology in T.annulata infection drawing on the work carried out by Sergent et al. (1945). The main signs are enlargement of the liver, spleen and lymph nodes. The abomasum shows characteristic necrotic ulcers which vary in size from 2.0 to 5.0 mm. The omasum is hard and its contents are, to a variable degree, dehydrated. Petechial haemorrhages can be observed on serous and mucous membranes but their presence on the epicardium is, usually, constant and a characteristic feature. Connective tissues become gelatinous and yellowish. According to Sergent et al. (1945) icterus was observed in one third of the cases which died of theileriosis and they observed, in a few cases, haematuria. According to the experience of the present author (unpublished work) the great majority of animals which died of artificially induced theileriosis due to strains of T.annulata showed icterus at postmortem examination but no case of haematuria or haemoglobinuria was observed.

Despite the interesting work of authors such as De Koch (1957), Barnett (1960) and Wilde (1966) on histopathological and haematological changes taking place in blood and bone marrow in T.parva infection, there is limited information, in this respect, in T.annulata infection. On histopathology, reference can be made to the brief description by Sergent, Donatien, Parrot Lestoquard, Palantureux and Rougebief (1924a). Haematological changes in T.annulata infection have also

been described by Sergent *et al.* (1929a, 1929b and 1945). These authors, while presenting the results of comprehensive studies carried out on the leucocyte changes in the course of the disease, very briefly described the changes in the red cells (Sergent *et al.* 1945). They noted anaemia, anisocytosis, poikilocytosis and punctate basophilia of the red cells in blood smears. On the changes occurring in the leucocyte numbers they gave the following account from a study carried out on 11 calves.

The total and differential leucocyte counts during the incubation period did not show any noticeable changes from the pre-infection picture. During the patent period leucocytosis occurred and the total leucocyte counts increased from 10,000 to 36,000, with an average of 19,000 per cubic mm. They attributed this leucocytosis to an increase in the number of lymphocytes. According to these authors leucocytosis was progressive and in parallel with the patent parasitaemia reaching its maximum at the time of death or the end of the reaction, when recovery started. They stated that leucocyte counts returned to normal values within 3 days of the termination of the thermal reaction.

Prasad (1946) from a limited number of experiments presented the results of haematological studies, in experimental *T.annulata* infection, which were in contrast with those of the previous authors. According to him a slight leucocytosis was observed during the incubation

period. The patent period was accompanied by leucopenia which persisted up to 2 or 3 days after termination of the reaction, when leucocyte counts returned to normal. He stated that leucopenia was due to the reduction in number of all the leucocytes except monocytes which became predominant. The following differential count during the patent period was given by him:

Lymphocytes	6%
Neutrophils	2%
Monocytes	91.5%
Eosinophils	0.5%

According to him, in fatal cases in extremis, the leucocytes while maintaining the percentages given in the above differential count showed leucocytosis. In one case the count was as high as 145,140 leucocytes per cubic mm. Prasad (1946) also referred to changes in the red blood cell counts and estimation of the haemoglobin during the course of experimental T.annulata infection. He considered these changes as being very slight. If the accuracy of the techniques he was using is not questionable, he might have been dealing with another disease as well as T.annulata infection.

TREATMENT

So far no investigator has introduced a drug with specific action on the causative agent of T.annulata infection. There are numerous reports of therapy with combinations of drugs. These successful reports should be considered in the light of the knowledge that in

T.annulata infection spontaneous recovery quite often occurs. Most of the authors who have claimed positive results in the treatment of T.annulata, have been dealing with natural theileriosis in the field. It is not very clear what would have been the rate of recovery, had treatment not been given at all. It is desirable that experiments on treatments should be carried out in groups of animals which are infected with a known strain of T.annulata. A review of the literature on the use of various compounds for treatment of T.annulata infection was carried out by Neitz (1957). Hawking (1958) published a list of drugs which he tested in tissue culture against macroschizonts of T.annulata and found none of the 40 compounds he used had any effect. Since the review by Neitz (1957) there have been many claims of successful treatment of T.annulata infection. A list of these references and the drugs used is presented in this thesis (Table 1). Pereponov and Nechaev (1963) claimed excellent results when they used primaquine which was followed by proguanil and symptomatic treatment. They mentioned that primaquine at the rate of 1 mg./kg. body weight if used in the early stage of the disease for 3 consecutive days had a specific effect on schizonts and proguanil at the rate of 12.5 mg./kg. body weight had a specific effect on the erythrocytic forms if used for 2 days. These authors (1964) again confirmed their previous results. Marutyan (1968) repeated the work of Pereponov and Nechaev (1964) and concluded that primaquine

Table 1

Drugs which have been tested in the treatment of T.annulata infection.

Drugs	Effect on the course of the disease	References
BABESICIDALS		
Berenil	±	Mahmoud, Haiba, Zafer and Awad (1956)
Berenil	-	Shmulevich and Evplov (1958)
Berenil	-	Pipano (1964)
Berenil	-	Giesecke and Wiesenhütter (1965)
Acaprine	-	Radkevich (1961)
Acaprine	-	Kardassis (1964)
Acaprine	-	Giesecke and Wiesenhütter (1965)
Bayer 9012	-	Giesecke and Wiesenhütter (1965)
ANTIMALARIAL		
Mepacrine	-	Kardassis (1964)
Proguanil	±	Kardassis (1964)
ANTIBIOTICS		
Chloramphenicol	-	Gayot (1957)
Oxytetracycline	±	Shmulevich and Evplov (1958)
BACTERICIDAL		
Acriflavine	-	Kardassis (1964)
BIOLOGICAL PRODUCTS		
Gamma globulin	+	Lavrentev (1958)

Table 1 (cont'd)

Drugs	Effect on the course of the disease	References
TREATMENT WITH A COMBINATION OF DRUGS		
Berenil and Acaprine	-	Shmulevich and Evplov (1958)
Biomycine (Chlortetracycline) and Berenil	+	Shmulevich and Evplov (1958)
Oxytetracycline and Berenil	+	Shmulevich and Evplov (1958)
Biomycine and Acaprine	+	Shmulevich and Evplov (1958)
Aminoacriquine, a solution of 10% Sodium bromide, Vitamin B12, Co Cl_2 and Cu SO_4	+	Dubovuyi (1958)
Chlortetracycline, Sulphantrol and symptomatic treatment	+	Shapochka (1958)
Tetracycline and Acaprine or Trypaflavine	+	Shmulevich and Evplov (1960)
Aminoacriquine, Chlortetracycline, a solution of 2% Procaine and Haemosporidin	±	Radkevich (1960)
Sulphantrol, a solution of 40% glucose, normal saline, Oxytetracycline, 2% Procaine solution, Aminoacriquine and Berenil	+	Radkevich (1960)
Chloroquine and Penicillin	+	Awad (1962)

Table 1 (cont'd)

Drugs	Effect on the course of the disease	References
Primaquine, Proguanil and symptomatic treatment	+	Pereponov and Nechaev (1963)
Primaquine, Proguanil and symptomatic treatment	+	Pereponov and Nechaev (1964)
Primaquine, Proguanil and symptomatic treatment	+	Marutyan (1968)
Amodiaquine hydrochloride (Camoquin) and Oxytetracycline	+	Narasimhamurty, Rajulu and Reddy (1970)

\- Not effective

\+ Beneficial effect

± Doubtful effect

had a specific action on schizonts but proguanil was effective only against the erythrocytic forms. The present author (unpublished work) at his laboratory, using a strain of T.annulata which did not produce erythrocytic forms, studied the effect of primaquine against the schizonts. Four infected calves received primaquine, orally, at the rate of 1 mg./kg. body weight for 5 days, commencing on the day of onset of fever. They were not given either proguanil or symptomatic treatment. The fever did not abate and all 4 calves died of acute theileriosis. This drug has also been tried against East Coast fever but was found to be ineffective (Wilde 1967; Snodgrass, Trees, Bowyer, Bergman, Daft and Wall 1972). These results indicated that the previous authors, who recommended primaquine, over estimated the specific effect of the drug and, most probably, the high percentages of recovery were due to other factors such as symptomatic treatment. Therefore, one is justified in accepting the conclusion of the thirtieth General Conference of the Committee of O.I.E. (1962) that there is, as yet, no satisfactory drug treatment for theileriosis.

CONTROL

Eradication of the disease can be achieved by the elimination of vector ticks, mainly by the use of acaricides, and/or by destroying the carrier animals. The prospect and the means of such eradication have been discussed by Neitz (1957) and Barnett (1968). Immunization

of susceptible cattle to reduce the mortality due to T.annulata infection has always been the aim of investigators. This method of control is feasible only when an effective vaccine is available. The effect of chemicals, serial passages of the parasite through susceptible animals and application of other means for the attenuation of T.annulata have been studied by Sergent et al. (1924a) and Sergent et al. (1927b and 1927c) and with the object of providing a vaccine. They found that serial passage in susceptible animals did not attenuate the parasite. Chemicals such as carbolic acid and formalin either did not affect the virulence or killed the parasite. Calves injected with formalin killed schizonts were not immunized and, on challenge, injected cattle were as susceptible as those which had not been injected. The authors also failed in their attempt to attenuate the organism by adding convalescent serum. When they used very small amounts of infected blood for vaccination the results, as they concluded later, were not constant and dependable (Sergent et al. 1945). Rafyi, Maghami and Hooshmand-Rad (1965) claimed the attenuation of T.annulata strains by serial passage in a breed of cattle, relatively, resistant to theileriosis. The present author, who followed up this work, found that these strains, when they were serially passaged in susceptible calves, became as virulent as before. The supposed loss of virulence was due, possibly, to the reduction in the number of schizonts in the circulating

blood which was used as the inoculum. Contrary to the work of Sergent et al. (1924a, 1927b and 1927c) Mirzabekov et al. (1969) claimed that he could attenuate the virulence of T.annulata strains by the addition of acridine, sodium sulphantrol and "Berenil"(4,4' -diamidino diazoaminobezene diaceturate)to infected spleen homogenized and suspended in normal saline solution. The animals inoculated did not show any untoward reaction and most of them were protected against subsequent challenge. The validity of this method remains to be tested.

Systematic studies on North African strains of T.annulata carried out in Algeria revealed that, in nature, there are strains of this parasite with low and even very low virulence. Sergent et al. (1945) isolated a strain, which they named "Kouba", that caused only 3% mortality in inoculated calves. This strain was maintained for nearly 12 years, by serial passage in cattle, and it was used as a vaccine strain for 10 years. From the eighteenth passage it failed to produce erythrocytic forms of the parasite and produced only macroschizonts. This strain and other strains of low virulence were intensively used in North African countries as well as in Israel for vaccination of cattle, especially young calves. According to Sergent et al. (1945) from 1924 to 1942 they vaccinated 36,631 head of cattle against T.annulata in Algeria, Tunisia and Morocco. Adler (1952) reported vaccination of approximately 6,000 to 8,000 calves each year in Israel. The method of vaccination

was by inoculation of 5 to 10 ml. of infected blood obtained from a calf during the period when macroschizonts could be detected in the liver or other organs. It was essential for this blood to be used within 3 days of the time of bleeding. Tsur (1965) stated that in Israel they were using 2 inoculations, the first with a very mild strain followed by a second, in 2 months time, with a virulent strain.

Sergent et al. (1945) claimed very good results with vaccination using a mild strain. According to these authors the following is a comparison of the losses in vaccinated and non-vaccinated herds.

Year	Losses in vaccinated herds %	Losses in non-vaccinated herds %
1933	0.9	9.5
1934	1.6	9.0
1935	0.8	5.2
1936	0.7	4.2
1937	1.9	56.7
1938	0.3	13.4
Mean	1	16.3

Adler and Ellenbogen (1936) stated that the Algerian vaccine strain "Kouba" gave satisfactory immunization against the virulent Palestine strains of T.annulata.

Delpy (1949) reported that in 1935 16, unvaccinated, grade bulls were imported into Iran. These bulls contracted theileriosis and 12 of them died within one month of their arrival. In 1936 5 grade bulls were

imported from Caucasia where they had been vaccinated. Four of these bulls contracted the disease but only one died. In 1939 48 cows were imported from France where they were vaccinated with the Algerian strain. After their arrival only 4 died of theileriosis, the rest remained healthy.

Sergent et al. (1945) concluded that once an animal has been vaccinated its immunity should be maintained by repeated reinfections. For this reason they suggested that animals should be vaccinated in early spring, before the season of abundant ticks, to provide protection against natural challenge infection in the field. These authors suggested that cattle stabled all the year round, should be revaccinated every year. Tsur, Hadani, Pipano, Cwilich, Senft and Cohen (1964) mentioned an outbreak of theileriosis with mortality in vaccinated herds. According to the authors mortalities were higher in those animals which had been vaccinated 3 to 4 years previously, indicating that immunity had waned since vaccination. They also suggested that vaccination should be repeated every year.

Although the use of blood vaccine decreases the incidence of clinical theileriosis and the mortality due to this disease, it has many disadvantages. The mild strain has to be kept by serial passage from animal to animal all the year round. Any intercurrent infectious disease means the risk of losing the vaccine strain. This method of maintenance of a vaccine strain is very

costly and laborious. The difficulties were alleviated to some extent when Tsur and Pipano (1962) were successful in preserving T.annulata schizonts at low temperatures. Even so, there is still the risk of transmission of other pathogens with vaccine prepared from the blood of an animal with intercurrent infection. Sergent et al. (1945) described methods for isolating T.annulata from other blood pathogenic protozoa but did not rule out the danger of such contamination.

TISSUE CULTURE

Tsur (Tchernomoretz) (1945) maintained viable schizonts of T.annulata for 12 days in an explant culture, using Tyrode solution enriched with calf serum, incubated at 38°C. However, there was neither growth nor multiplication. In later publications Tsur (1947 and 1953) confirmed his previous report that the addition of growth factors such as glutamine, pyridoxine, riboflavine and inositol to the medium resulted in the growth of the schizonts. This growth was observed for 2 months in 10 successive fragments of normal calf spleen. The author stated that the schizonts remained infective for calves, after 4 passages. It was also shown that schizonts could grow in juxtaposition to spleen fragments from recovered animals or in medium enriched with 40% serum of animals recovered from theileriosis. Brocklesby and Hawking (1958) repeated this work and were able to grow the schizonts of T.annulata. They, for the first time, expressed doubts about the then current view that the schizonts break up

into merozoites which infect fresh cells. These authors, by taking into consideration the mode of multiplication of Plasmodium gallinaceum and P.relictum in tissue culture, postulated that if this phenomenon takes place with T.annulata, it must be exceptionally rapid.

Tsur and Adler (1962) reported the cultivation of T.annulata schizonts in monolayers prepared from the spleens and other organs of infected calves. These authors stated that the schizonts could multiply in the cultures without the addition of fresh lymphoid cells, but did not try to give any explanation as to how these schizonts were propagated in the culture nor as to the source of fresh uninfected lymphoid cells. Light was thrown on this question by Hulliger, Wilde, Brown and Turner (1964) when they published their paper on the mode of multiplication of the schizonts of T.parva in lymphoid cells. They explained for the first time how multiplication of theilerial schizonts is synchronized with the division of the host lymphoid cells. Hulliger (1965) published another paper on cultivation of three species of Theileria in tissue culture and threw more light on the propagation of Theileria spp. in tissue culture. This author cultivated lymphoid cells containing schizonts in association with baby hamster kidney (BHK) cells and suggested that for growth and propagation of infected lymphoid cells, the presence of fibroblast cells as a feeder layer was needed. Zablotskyi (1966 and 1967) also reported the successful cultivation

of T.annulata in tissue culture and discussed the possibility of using it as a means for studying the parasite. Hooshmand-Rad and Hashemi-Fesharki (1968) for the first time cultivated lymphoid cells containing T.annulata schizonts in suspension culture, independent of a fibroblast feeder layer.

TISSUE CULTURE VACCINE

Tsur (1965) suggested the possibility of using tissue cultured schizonts of T.annulata for vaccination of susceptible animals. Pipano and Tsur (1966) published the results of laboratory trials for the vaccination of susceptible calves, against T.annulata infection. Tsur and Pipano (1966) described their work on attenuation of a T.annulata strain after consecutive passages in tissue culture. Attenuation of the strain progressed with in vitro passages and a point was reached when the parasite did not provoke a thermal or parasitic reaction in inoculated young and adult cattle. According to these authors all the inoculated animals were found to be immune to subsequent challenge with a virulent strain. The ~~inoculated~~ control animals suffered a mortality of 31%. They failed to mention whether the challenge strain was homologous or heterologous. Rafyi et al. (1965) had shown that strains of T.annulata produced a complete and solid immunity against the homologous strain but only a partial immunity against heterologous strains.

Pipano (1970) in his experiment to immunize calves with an attenuated strain, stated that in order to

correlate the immunity status of his experimental calves with serological findings he had used a virulent homologous strain. The author of this thesis does not consider this method of challenge a suitable means of determination of the true immune status of an animal.

Pipano and Cahana(1968) applied the indirect fluorescent antibody technique for detection of the animals' response to T.annulata tissue culture vaccine, as the strain they used was so attenuated that it did not provoke a thermal or parasitic reaction. Hooshmand-Rad and Hashemi-Fesharki (1971) applied the complement fixation test in the detection of response to vaccination with an attenuated strain. They found this method was quite reliable in detecting the response of animals, although they could not correlate the degree of serological reaction with the immune status. They suggested that the best time for collecting sera for the test was the 40th day after vaccination.

PRESERVATION

Tsur and Pipano (1962) showed that schizonts of T.annulata in blood or suspensions prepared from spleens and livers could be preserved at approximately -70°C. in the presence of 15% glycerol as the cryoprotectant. They showed that schizonts stayed viable even after 150 days in these conditions. Tsur et al. (1964) briefly stated that they could provoke theileriosis by inoculating lymphoid cells containing T.annulata schizonts which had been kept at -70°C. for up to 20 days. Hulliger (1965)

prepared stabilates of Theileria infected lymphoid cells and preserved them at -79°C. in the presence of 10% glycerol. Rafyi, Maghami and Hooshmand-Rad (1967) reported that T.annulata schizonts could be preserved for at least 277 days.

This review of literature reveals that the information on T.annulata tissue culture vaccine is very limited. The few papers which are available on this subject do not present substantial information on the preparation and the application of a vaccine and the matter is dealt with in a brief and superficial manner. The economic importance of the disease demands more effort to be put into investigations with the object of improvement and enhancement of the effectiveness of such a vaccine. The chapters to follow will present the results of the attempts of the author of this thesis to standardize the methods of preparation of vaccine, vaccination and determination of the duration of the immunity engendered by the administration of such a standardized vaccine.

Chapter 3

MATERIALS AND METHODS

1 - Experimental cattle

The calves and heifers used in these experiments which were conducted in Baghdad were all of Friesian breed, their dams having been imported from France, and were considered to be very susceptible to T.annulata infection. The calves were from 4 to 12 months and the heifers from 16 to 18 months of age. They were raised on a farm where strict tick control measures were practised. During the seasons when ticks were abundant the animals were sprayed (the calves by hand and the heifers in a spray race) twice weekly with acaricides, Diothyl (I.C.I.) and Neocidol (Geigy) at the dilutions recommended by the manufacturers. This method, apparently, was efficient in keeping the animals tick-free, as in the course of the experiments neither tick infestation nor natural theileriosis were observed.

2 - Strains of T.annulata

Strain No. 15 (referred to in the present work as Strain A). This Strain had been isolated in tissue culture from the prescapular lymph node of a calf in which T.annulata infection had been artificially induced by blood passage in Iran at the Razi Institute. It was used at the sixty sixth tissue culture passage for vaccine production but also was passaged further and studied for attenuation up to 230 passages in tissue culture.

Strain No. 11 (referred to in the present work as Strain B). This Strain also had been isolated in tissue culture from the spleen of a calf in which T.annulata infection had been artificially induced at the Razi Institute. A stabilate of this strain which had been stored at low temperatures for 4 years was revived at the beginning of the present work. This is the longest period of cold storage of T.annulata infected lymphoid cells so far reported.

Strain C. This Strain was isolated in tissue culture from the lymph node of a calf naturally infected with T.annulata infection, in Baghdad. It was also isolated by transfusion of infected blood to susceptible cattle and was used as the challenge strain in the first challenge test.

Strain D. This Strain was isolated from a fatal case of natural T.annulata infection, in Baghdad. It was also isolated in tissue culture as well as by blood passage. Infected blood was preserved at -70°C. and the strain was used in the second challenge test.

The method of isolation of Strains C and D in tissue culture was as described by Hooshmand-Rad and Hashemi-Fesharki (1968), but the nutrient medium, Eagle's, was enriched with lactalbumin hydrolysate and yeast extract. The amounts of these substances per litre of Eagle's medium and the work which led to the selection and the determination of them will be described later. The lymphoid cells containing schizonts of T.annulata (infected

lymphoid cells), using the modified Eagle's medium, grew faster than fibroblasts and established themselves in suspension culture in 7 days, whereas at that stage of culture fibroblasts had just begun to grow.

3 - Observations on cattle

(i) - Body temperature recording. Rectal body temperatures of the experimental animals were recorded in the morning. Elevation of the temperature to 103°F. or above was considered as fever.

(ii) - Blood smears. Blood smears were prepared in the conventional manner with blood obtained by puncture of an ear vein. They were also prepared from blood samples drawn for haematological studies. New slides with soft ground edges were used. These were soaked in 95% ethyl alcohol overnight, dried and polished with a lint-free cloth and stocked in clean boxes until use. Air dried smears were fixed by placing them for 3 minutes in absolute methyl alcohol. Staining was carried out with Giemsa stain (Gurr R 66). A solution of 5% Giemsa stain in a buffer (pH 7.0) was used. The smears were allowed to stain for 45 minutes.

(iii) - Lymph node biopsy. The skin at the site of the puncture was shaved and was swabbed at first with diluted tincture of iodine and then the iodine was washed off with 70% ethyl alcohol. The lymph nodes were secured under the skin by manual pressure. A needle of gauge 20 and length of, approximately, 5 cm. was inserted

into the node. The upper orifice of the needle was blocked by applying the finger tip while withdrawing the needle. The fluid in the lumen of the needle was transferred to a clean glass slide and with the aid of another glass slide thin smears were prepared. Fixation and staining were carried out as described for blood smears.

(iv) - Liver biopsy. The liver biopsy was carried out as described by Sergent et al. (1924b) except that incision of the skin, prior to insertion of the needle, was not practised as the skin of the animals used in the experiments was thin and did not present undue resistance to the insertion of the needle. Fixation and staining methods were as described for blood smears.

4 - Haematology

Blood for haematological examinations was collected in vials containing the disodium salt of ethylenediamine-tetra-acetic acid (EDTA). The vials containing blood were placed on a cell suspension mixer prior to sampling. Haematological procedures were carried out in the following order.

(a) Preparation of blood smears - immediately after bleeding.

(b) Estimation of PCV - within 2 hours of bleeding.

(c) Total red and white cell counts - within 5 hours of bleeding.

(d) Estimation of haemoglobin - within 30 hours of

bleeding, blood being preserved at 4°C. meanwhile.

(e) Differential white cell counts - at any convenient time.

(i) - Packed cell volume (PCV). This was determined with a microhaematocrit centrifuge (ADAMS Autocrit Centrifuge). Capillary tubes were sealed by using plasticine, supplied with the centrifuge set, and were centrifuged for 15 minutes. The reader on the centrifuge head was used to estimate the percentage volume.

(ii) - Haemoglobin concentration. This was estimated using a Medical Research Council (MRC) photometer (Keeler Optical Products) as described by King et al. (1948), based on and calibrated for estimation of haemoglobin by the oxyhaemoglobin method. A 0.5% dilution of blood in ammoniated distilled water was compared with distilled water by using Filter Cap No. 2 (Ilford 625) and artificial illumination.

(iii) - Red cell counts. These were made by the visual method (Schalm 1965). An improved Neubauer haemocytometer was used. Blood was diluted 1 in 200, with a solution of 0.9% sodium chloride, in diluting pipettes.

(iv) - Total leucocyte counts. These were carried out as described by Schalm (1965). The blood was diluted 1 in 20, in a solution of 2% acetic acid which was tinted with a 1% aqueous solution of gentian violet. An improved Neubauer haemocytometer was used. Only counts

which did not show more than 10% difference in the counts of the 2 chambers were accepted.

(v) - Differential leucocyte counts. Two blood smears were prepared from each blood sample. The date and the number of the sample were engraved on the glass slide using a diamond marker. Smears were fixed and stained as described above. The battlement method (Schalm 1965) was used for counting the leucocytes.

5 - Serology

The indirect fluorescent antibody (IFA) test.

(a) Antigen. Schizonts of T.annulata propagated in suspension culture of lymphoid cells were used as antigen. Four day old cultures containing approximately 8×10^5 cells/ml. were centrifuged and the sedimented cells were washed twice with phosphate buffered saline (PBS) pH 7.0. Sedimented cells at the last washing were resuspended in PBS containing 5% powdered bovine plasma albumin fraction V (Nutritional Biochemical Corporation, Cleveland, U.S.A.) to make a concentrated suspension. Thin smears from this suspension were prepared on glass slides and allowed to dry in air. The smears were fixed for 10 minutes in acetone and then were wrapped in thin paper and were stored at $-70^{\circ}C$. until required.

(b) Conjugate. The conjugate was rabbit anti-bovine gamma globulin conjugated with fluorescein isothiocyanate (Nutritional Biochemical Corporation, Cleveland, U.S.A.). It was diluted in PBS and a dilution that gave maximum fluorescence with a positive serum,

from a calf having recovered from natural theileriosis, and did not give fluorescence with the negative serum, was taken as the optimum dilution. This was found to be 1 in 20.

(c) Control negative sera. The pre-inoculation sera of animals to be tested were considered as negative sera. Serum from a 4 months old uninfected calf was also used as a control negative serum. The control sera were tested at a dilution of 1 in 4 in PBS.

(d) Test sera. Blood was collected, by jugular puncture, in Universal bottles. After one hour at room temperature and 2 hours at 37°C., serum was seperated from the blood by centrifuging at 3,000 r.p.m. for 20 minutes. Sera were preserved at -22°C. until required. They were tested using 2 fold dilutions in PBS starting with a dilution of 1 in 4.

(e) Staining procedure. Slides were removed from storage at -70°C. and were left at 4°C. for 30 minutes and then were unwrapped and kept for 30 minutes at room temperature. The backsof slides were marked with circles to delineate 10 areas for negative and test sera, then they were placed in a moist chamber. One drop of the pre-inoculation serum of each calf at the dilution of 1 in 4 was pipetted into the first circle. One drop of each of 8 dilutions of the test serum from the same calf were pipetted into the respective circles. One drop of the control negative serum at the dilution of 1 in 4 was pipetted into the last circle. The slides were incubated

at 37°C. for 30 minutes, after which the sera were washed off with PBS by 2 15-minutes rinses in fresh PBS in a jar containing a magnetic stirrer. They were dried in air and then one drop of the optimum dilution of the conjugate was pipetted into each circle. The incubation, washing and drying were repeated once more as described above. The slides were mounted for examination by placing a drop of a solution of 50:50 glycerol and PBS v/v and a cover-glass on each circle.

(f) Microscopy. An Olympus microscope which was equipped with a HBO 200 watt pressure mercury vapour lamp, the necessary filters and a dark-field condenser was used. The exciting filter and the objective used were BG 12 and oil immersion x 40, respectively. The fluorescence was graded as, shiny fluorescent + + + +, fluorescent + + + and hazy fluorescent + +. The titre of the serum was regarded as the highest dilution showing + + + fluorescence.

6 - Tissue culture techniques

(a) Cleaning and preparation of glassware.

Glassware (except pipettes), rubber bungs, rubber tubing and plastic caps were washed using a detergent, Microsolve (Microbiological Associates of Bethesda, U.S.A.). They were rinsed several times under tap water and once in distilled water. Pipettes were washed by placing them in a solution of chromic acid and rinsing them with tap and distilled water. After drying they were prepared for sterilization.

(b) Sterilization.

(i) - Dry heat. All glassware was sterilized at 160°C. for 2 hours in a dry heat oven.

(ii) - Moist heat. All articles made of rubber, glassware carrying rubber parts and Seitz filters were sterilized in an autoclave at 15 lb./sq.in. for 30 minutes. Salt solutions with the exception of the solution of sodium hydrogen carbonate were sterilized at 15 lb./sq.in. for 30 minutes. Solutions of sodium hydrogen carbonate were sterilized at 10 lb./sq.in. for 15 minutes.

(iii) - Seitz filtration. All the heat labile materials such as serum or amino-acids were Seitz filtered.

(c) Preparation of serum, reagents and media.

(i) - Serum. Blood from calves slaughtered in the city abattoir was collected and serum was separated. It was preserved at -22°C. prior to use.

(ii) - Phosphate buffer saline without calcium and magnesium. The following solution was prepared.

Na Cl	40.00	g.
K Cl	1.00	g.
$Na_2H\ PO_4,\ 2H_2O$	5.75	g.
KH_2PO_4	1.00	g.
Double distilled water to make	1,000	ml.

This solution was diluted with 5 times its volume of double distilled water at the time it was used.

(iii) - Hanks' balanced salt solutions (BSS).

Solution (A) x 10

Na Cl	80.00	g.

K Cl	4.00	g.
$Mg\ SO_4, 7\ H_2O$	1.00	g.
$Na_2HPO_4, 2\ H_2O$	0.60	g.
KH_2PO_4	0.60	g.
Glucose	20.00	g.
Phenol red	200.00	mg.
Double distilled water	1,000	ml.

Solution (B) x 10

$Ca\ Cl_2$	1.40	g.
$Mg\ Cl_2, 6H_2O$	1.00	g.
Double distilled water	1,000	ml.

Solution (C)

$Na\ H\ CO_3$	44.00	g.
Double distilled water	1,000	ml.

These solutions after being thoroughly mixed were dispensed in exactly 100 ml. amounts in prescription bottles and were sterilized by autoclave and were stored at $+4^{\circ}C$.

(iv) - Antibiotics. Penicillin and streptomycin were used in media at the rate of 100 units/ml. and 100 ug./ml. respectively. An aqueous solution was made to contain 10^5 units/ml. of sodium penicillin G and 10^5 ug./ml. streptomycin. This solution was sterilized by Seitz filtration and stored at $-22^{\circ}C$.

(v) - Stock solutions of amino-acids and vitamins for preparation of Eagle's medium. The formulae described by Eagle (1955 and 1959) were compared. The latter showed no advantage over the former but the amounts

of the ingredients were almost 10 times greater and, therefore, more costly, for which reason the earlier formula was used.

Stock solution of amino-acids No. 1 (x 100). The following amounts of L-amino-acids were dissolved in 500 ml. double distilled water by heating to 80°C.

Arginine	0.870 g.
Histidine	0.170 g.
Lysine	0.940 g.
Leucine	0.655 g.
Iso-leucine	1.310 g.
Methionine	0.375 g.
Phenylalanine	0.415 g.
Threonine	0.595 g.
Tryptophan	0.600 g.
Valine	0.585 g.

Stock solution of amino-acids No. 2 (x 100). The following L-amino-acids were dissolved in 500 ml. of a solution of 0.1 N H Cl in double distilled water.

Tyrosine	0.90 g.
Cystine	0.30 g.

Stock solution of amino-acid No. 3 (x 100). The following amino-acid was dissolved in 500 ml. double distilled water.

Glutamine	7.30 g.

Stock solution of vitamins No. 1 (x 1,000). The following B vitamins were dissolved in 500 ml. of double distilled water.

Choline	0.50 g.

Nicotinic acid	0.50 g.
Pantothenic acid	0.50 g.
Pyridoxal	0.50 g.
Riboflavine	0.05 g.
Thiamine (aneurin)	0.50 g.
Inositol	0.50 g.

Stock solution of vitamins No. 2 (x 100). The following B vitamins were dissolved in 500 ml. double distilled water by the addition of a few drops of 0.5 N Na OH.

Biotin	0.05 g.
Folic acid	0.05 g.

These stock solutions were sterilized by Seitz filtration. They were dispensed in 20 ml. amounts and stored at -22°C.

(vi) - Preparation of Eagle's medium based on Hanks' BSS. For preparing one litre of Eagle's medium, double distilled water and the stock solutions were poured into a volumetric flask, containing a magnetic stirrer, in the following sequence:-

Double distilled water	200 ml.
Hanks' BSS (A) x 10	100 ml.
Hanks' BSS (B) x 10	100 ml.
Stock solution of amino-acids No. 1	10 ml.
Stock solution of amino-acids No. 2	10 ml.
Stock solution of amino-acid No. 3	10 ml.
Stock solution of vitamins No. 1	1 ml.
Stock solution of vitamins No. 2	10 ml.
Calf serum	100 ml.
Stock solution of antibiotics	1 ml.

Hanks' BSS (C)	8 ml.
Double distilled water to make	1,000 ml.

The flask was stoppered and placed on a magnetic mixer for 15 minutes. The medium was cultured on PPLO growth medium (Difco) and 100 ml. were placed in an incubator at 37°C. for 96 hours to test the sterility.

(d) Culture of infected lymphoid cells.

Cultivation of infected lymphoid cells was carried out according to the method of suspension culture described by Hooshmand-Rad and Hashemi-Fesharki (1968) using Eagle's medium. The rate of growth of infected lymphoid cells by this method was relatively low. Experience had shown that infected lymphoid cells which were grown in association with autologous, homologous or heterologous fibroblast cells (mixed cultures) had a higher rate of growth. It seemed that the infected lymphoid cells in mixed cultures had access to some required substance, possibly resulting from metabolism of the fibroblasts, which was insufficient in Eagle's medium. An event, which will be described below, led to some modification of the nutrient medium which resulted in obtaining a faster rate of growth of infected lymphoid cells.

During subculture of infected lymphoid cells there was a shortage of Eagle's medium for one culture bottle. The nutrient medium in this bottle was supplemented with an equal quantity of Hanks' BSS enriched with 0.5% lactalbumin hydrolysate and 0.1% yeast extract (Hanks'LY).

Routine daily microscopic checks of the cultures revealed, unexpectedly, a higher and better growth in the culture bottle with mixed medium. This finding was quite surprising as in previous trials Hanks' LY had proved to be unsuitable for the growth of infected lymphoid cells in mixed as well as in suspension cultures. After repeating this, it was proved that a mixture of Eagle's and Hanks' LY media resulted in faster growth. With good reason this increase in the rate of growth was assumed to be due to the presence of lactalbumin hydrolysate and yeast extract (LY) in the medium. To investigate the effect of LY and determine the optimum amount of these substances required in the Eagle's medium, the following experiment was carried out.

Experiment 3.1

A stock solution containing 25 g. lactalbumin hydrolysate (Nutritional Biochemical Corporation, Cleveland, U.S.A.) and 5 g. yeast extract (Difco) were dissolved in one litre of double distilled water and sterilized by autoclaving. Eagle's media enriched with different quantities of this solution, beginning with 10 ml. and increasing by 10 ml. up to 50 ml., were prepared. At successive intervals, cultures with 10^5 infected lymphoid cells were seeded, each time using one of the media described above. Cells were counted immediately after implantation and thereafter every 24 hours. The growth rates of the infected lymphoid cells in each of the 5 media were determined and were compared against the rate of

growth of those cells grown in Eagle's medium without supplement.

The results are presented in Table 2 and Fig. 1. Cells in ordinary Eagle's medium increased 4 fold after 96 hours incubation. The growth rates of infected lymphoid cells, cultured in Eagle's medium containing LY, were higher than those cultured in ordinary Eagle's medium (Table 2). The duration of growth and the increase in the number of cells/ml. correlated positively with the amounts of LY in the medium, up to 40 ml./litre. Infected lymphoid cells cultured in the medium containing 40 ml. or 50 ml./litre of LY stock solution multiplied by almost 9 fold in 96 hours and the growth curve acquired a sygmoid form (Fig. 1).

On the basis of these results a slight modification in the formula of Eagle's medium was made. The definitive medium used was, therefore, as follows:

Double distilled water	200 ml.
Hanks' BSS (A) x 10	100 ml.
Hanks' BSS (B) x 10	100 ml.
Stock solution of amino-acids No. 1	10 ml.
Stock solution of amino-acids No. 2	10 ml.
Stock solution of amino-acids No. 3	10 ml.
Stock solution of vitamins No. 1	1 ml.
Stock solution of vitamins No. 2	10 ml.
Calf serum	100 ml.
Stock solution of LY	40 ml.
Stock solution of antibiotics	1 ml.

Table 2

Growth rate of infected lymphoid cells cultured in Eagle's medium supplemented with 10% calf serum and various amounts of LY stock solution*.

Incubation Period (hours)	Millilitre of LY/litre Eagle's medium					
	0	10	20	30	40	50
0	100	100	100	100	100	100
24	114	130	128	122	132	156
48	180	254	258	340	468	414
72	290	334	478	772	714	736
96	380	420	520	610	886	892
120	280	380	490	580	814	804

Figures in columns 2 to 7 represent the number of infected lymphoid cells/ml. ($\times 10^3$) and are the mean counts of four cultures.

* A solution of 25 g. lactalbumin hydrolysate and 5 g. yeast extract in one litre of double distilled water.

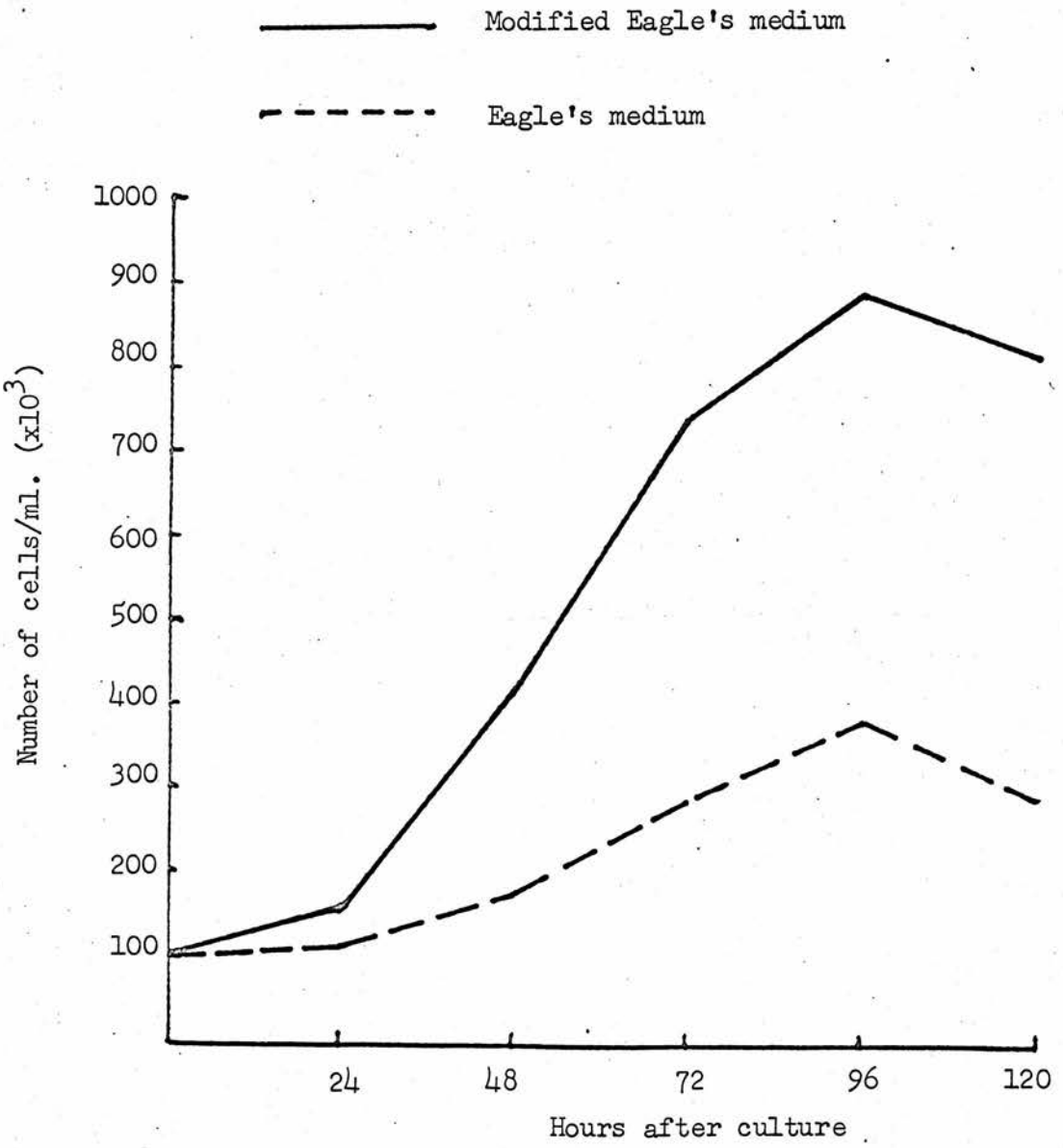

Fig. 1 Growth curves of infected lymphoid cells in Eagle's medium and modified Eagle's medium.

Hanks' BSS (C)	8 ml.
Double distilled water to make	1,000 ml.

This formula was used for cultivation of infected lymphoid cells and henceforth will be referred to as modified Eagle's medium or nutrient medium.

The following 2 experiments were carried out to investigate the effects of the number of cells/ml. originally implanted and variation in serum content, on the growth of infected lymphoid cells.

Experiment 3.2

This experiment was designed to investigate the effect of the number of cells, originally implanted, on the rate of growth. This was carried out using the 2 types of Eagle's medium. Cultures with 10^4, 5×10^4, 10^5 and 2×10^5 cells/ml. were made using Eagle's original medium. Cultures with 10^4, 10×10^4, 10^5, 2×10^5, 4×10^5 and 8×10^5 cells/ml. were made using modified Eagle's medium as described above. Cells were counted immediately after culture and thereafter every 24 hours.

The results are given in Table 3. Cultures with Eagle's medium did not establish themselves if less than 10^5 infected lymphoid cells/ml. were originally implanted. Cultures with modified Eagle's medium were established even when 10^4 infected lymphoid cells/ml. were originally implanted. The times needed for cultures, with modified Eagle's medium, to reach to the peak were 8 and one days when 10^4 and 8×10^5 cells/ml. were originally implanted respectively.

Table 3

Growth of infected lymphoid cells in Eagle's medium and modified Eagle's medium when various numbers of cells were implanted in cultures.

Medium	Cells*/ml. cultured ($\times 10^3$)	Maximum growth observed. Cells*/ml. ($\times 10^3$)	Time for maximum growth (days)
Eagle's	10**	-	-
"	50**	-	-
"	100	380	4
"	200	420	2
Eagle's (modified)	10	833	8
"	100	892	4
"	200	820	3
"	400	980	2
"	800	1060	1

* Intact cells as judged by microscopic examination.
Figures represent the mean counts of four cultures.
** Cultures could not be established.

Table 4

* Growth of infected lymphoid cells in modified Eagle's medium enriched with calf, sheep and horse serum.

Serum	Cells/ml. (x 10^3) in cultures after				
	0	24	48	72	96 (hours)
Calf	100	185	390	770	840
Sheep	100	154	409	780	802
Horse	100	132	290	607	782

* Growth characteristics of the infected lymphoid cells did not alter even after 20 subcultures in modified Eagle's medium enriched with sheep or horse serum.

Figures represent the mean counts of four cultures.

Experiment 3.3

This experiment was carried out to compare, firstly, the effect of calf's serum at 10% and 20% levels in the nutrient medium, and secondly, the effect of 10% sheep or horse serum in the nutrient medium, on the growth of infected lymphoid cells. Cultures of 10^5 infected lymphoid cells/ml. in the nutrient media containing each of the above mentioned sera were made. Cells were counted immediately after the setting up of the cultures and thereafter every 24 hours.

The results are given in Table 4. There were no measurable changes resulting from using the above mentioned sera. Successive subcultures using sheep and horse sera did not alter the growth characteristics of infected lymphoid cells.

7 - Attenuation of T.annulata strains in tissue culture.

Attenuation, by one means or another, is desirable to render a pathogenic micro-organism harmless, in order to be used as a live vaccine. Some pathogenic micro-organisms show a degree of correlation between their pathogenicity and immunogenicity, while others do not. The attenuation of a pathogenic micro-organism is not successful unless its immunogenicity is preserved. Tsur and Pipano (1966) stated that a strain of T.annulata had been rendered completely attenuated after being in tissue culture for nearly 20 months. They claimed that animals inoculated with this strain showed neither thermal nor

parasitic reactions but they were found to be resistant to challenge with a virulent strain. This was an indication that immunogenicity of T.annulata strains was preserved after they were considerably attenuated. In this thesis the courses of attenuation of four strains of T.annulata, namely, Strain A, Strain B, Strain C and Strain D are described. The criteria for assessing the degree of attenuation were clinical and parasitological reactions.

(i) Strain A.

There was some degree of attenuation up to the fifty fourth passage (279 days) but thereafter there was no further attenuation of this strain even after 230 passages (1110 days). Although schizonts and fever were noted in the inoculated animals, the erythrocytic forms of the parasite did not appear in the blood smears of calves that had received the schizonts which had been 279 or more days in tissue culture (Table 5).

(ii) Strain B.

The course of attenuation in this strain was continuous and progressive, in parallel with the time of in vitro culture, and eventually this strain was rendered completely inocuous after 24 passages (110 days) in tissue culture. The calves that received, by different routes, various numbers of schizonts which had been for 110 days or more in tissue culture showed neither parasitic nor thermal reactions (Table 6).

(iii) Strain C.

This strain caused severe reactions in the early

passages but gradually became attenuated. The 2 calves that received 3 x 10^6 schizonts of this strain which had been in tissue culture for 56 passages (240 days) showed no parasitaemia but did undergo a slight thermal reaction (Table 7).

(iv) Strain D.

The reaction caused by this strain in inoculated animals was different from that of the other strains. The reactions caused by the schizonts of this strain, which had been in tissue culture for a few days, were very mild. It seemed, there were no alterations in the degree of the virulence, which was very mild, in the course of the tissue culture passages. One calf and 8 heifers that received the schizonts which had been in culture for 38 passages (165 days) showed mild reactions (Table 8).

8 - Storage of infected lymphoid cells.

The object of this work was to select the best method of preserving cell lines (infected lymphoid cells), seed and vaccine. Since freeze drying, which is the method of choice for preservation of viruses and bacteria, cannot be applied to biological cells, the applicability of the following methods was studied.

(1) - Storage at ambient temperature (26 - 35°C.):-

(a) In the presence of glycerol.

(b) Without the addition of glycerol.

(2) - Storage at +4 - +8°C.

(a) In the presence of glycerol.

Table 5

Course of attenuation of Strain A during 1110 days of <u>in vitro</u> culture.

Days in culture	Number of schizonts inoculated ($\times 10^6$)	Route of inoculation	Number of animals	Schizonts in Liver	Schizonts in Lymph node	Time in days to Fever	Time in days to Schizonts	Max. temp. °F.	Erythrocytic forms %	Reaction
19	7	s/c	1[x]	+	+	6	3	105.0	0.4	Moderate
48	3	s/c	1	+	+	8	5	107.2	2.0	Severe
132	3	s/c	1	+	+	4	2	104.6	-	Mild
157	3	s/c	2	+	+	2	2	105.2	0.1	"
				+	+	4	3	105.1	-	"
174	3	s/c	1	+	+	3	3	105.2	0.1	"
279	3	s/c	1	-	+	2	2	103.2	-	"
433	3	s/c	1	+	+	1	4	103.7	-	"
550	3	s/c	1	+	+	5	5	105.0	-	"
920	3	s/c	4	+	+	3	3	104.8	-	"
				+	+	2	3	105.2	-	"
				+	+	2	1	104.6	-	"
				+	+	3	3	105.2	-	"
1110	3	s/c	4	+	+	2	2	105.2	-	"
				+	+	1	1	104.9	-	"
				+	+	3	2	104.9	-	"
				+	+	3	3	105.4	-	"

x Local breed. - = Negative.

Table 6

Course of attenuation of Strain B during 140 days of *in vitro* culture.

Days in culture	Number of schizonts inoculated ($\times 10^6$)	Route of inoculation	Number of animals	Schizonts in: Liver	Schizonts in: Lymph node	Time in days to: Fever	Time in days to: Schizonts	Max. temp. °F.	Erythrocytic forms %	Reaction
17	9	s/c	1[x]	+	+	3	3	104.0	0.2	Mild
48	3	s/c	1	+	+	3	3	105.2	0.2	"
65	3	s/c	1	+	+	4	2	104.2	0.4	"
83	3	s/c	1	-	+	2	3	104.0	1.0	"
110	3	s/c	12	-	-	1	-	-	-	-
110	12	i/v	1	-	-	1	-	103.1	-	-
140	3	s/c	2	-	-	-	-	-	-	-

[x] Local breed. - = Negative.

Table 7

Course of attenuation of Strain C during 240 days of <u>in vitro</u> culture.

Days in culture	Number of schizonts inoculated ($\times 10^6$)	Route of inoculation	Number of animals	Schizonts in: Liver	Schizonts in: Lymph node	Time in days to: Fever	Time in days to: Schizonts	Max. temp. °F.	Erythrocytic forms %	Reaction
48	3	s/c	1	+	+	7	6	106.2	4.0	Severe
124	3	s/c	1	+	+	5	4	105.2	0.1	Mild
160	3	s/c	2	*	+	1	1	104.0	0.2	Very Mild
				*	+	1	1	103.2	0.2	
240	3	s/c	2	-	-	2	-	103.6	-	No parasitic reaction
				-	-	2	-	103.4	-	

* = Not carried out. - = Negative.

Table 8

Course of attenuation of Strain D during 165 days of <u>in vitro</u> culture.

Days in culture	Number of schizonts inoculated ($\times 10^6$)	Route of inoculation	Number of animals	Schizonts in lymph node	Time in days to Fever	Time in days to Schizonts	Max. temp. °F.	Erythrocytic forms %	Reaction
4	7	s/c	1*	+	1	5	104.0	0.7	Very mild
40	3	s/c	2*	-	3	-	104.2	0.5	"
				+	3	3	105.2	1.0	"
90	3	s/c	1*	+	2	1	103.6	0.3	"
165	3	s/c	1*	+	-	2	-	Rare	"
165	3	s/c	8**	+	2	2	103.1	"	"
				+	4	1	103.6	"	"
				+	3	1	103.6	"	"
				+	2	2	105.2	"	"
				+	3	1	103.6	"	"
				+	2	2	105.2	"	"
				+	3	2	104.0	"	"
				+	3	2	105.3	"	"

- = Negative. * Calves (4 to 6 months old). ** Heifers (18 months old).

(b) Without the addition of glycerol.

(3) - Storage at -22°C. in the presence of 10% glycerol.

(4) - Storage at low temperatures (-70°C.). In connection with this method the following factors were studied:-

(a) The comparison of cryoprotectants and the methods of eluting them from the cells.

(b) The effect of the rate of cooling.

(c) The effect of equilibration time.

Materials

Cells. Infected lymphoid cells, Strain A, were used. The nutrient medium was used to prepare suspensions of infected lymphoid cells.

Containers. Suspensions of cells were dispensed in vials, of 20 ml. capacity, which were sealed with rubber and aluminium caps.

Cryoprotectants. Pure neutral glycerol and dimethyl sulphoxide (DMSO) were the cryoprotectants used in this experiment. Glycerol was studied at 7.5%, 10% and 15% levels and DMSO at 10% level.

Deep freeze. For -22°C. an ordinary deep freeze cabinet and for -70°C. a Revco deep freeze cabinet were used.

Criteria for detection of cell viability.

(i) - Counting. An improved Neubauer haemocytometer was used. Only undamaged cells with characteristic refraction were counted.

(ii) - Tissue Culture. Counting of the undamaged cells,

alone, was not a satisfactory means for checking the percentages of the viable schizonts in the cells. As only lymphoid cells with viable schizonts can propagate, a certain number of infected lymphoid cells were implanted in culture bottles and multiplication was checked every 24 hours to correlate the results obtained from counting with the viability of cells and schizonts. The number of the viable cells in the implant was estimated from the number of cells/ml. after 48 hours culture.

Methods of freezing and thawing.

(i) - Freezing. The slow methods, controlled (1°C./minute) and uncontrolled, were used.

(ii) - Thawing. This was carried out by the rapid method (placing the vials in a waterbath at 40°C.).

Elution of the cryoprotectants.

This was carried out in 2 ways.

(a) The rapid method. Suspensions were centrifuged down, the supernatant fluid was discarded, fresh culture medium was added and the cells were resuspended.

(b) The slow method. Suspensions were centrifuged down and the supernatant fluid was discarded. An equal volume of the nutrient medium was added, half the volume drop by drop and the other half somewhat faster, while gently shaking the centrifuge tubes or the containers. Another volume of nutrient medium was added. The suspensions were thoroughly mixed and then centrifuged. The supernatant fluid was discarded and nutrient medium was added to the sedimented cells to resuspend them.

Experiment 3.4

Suspensions of infected lymphoid cells were prepared in the nutrient medium and the nutrient medium containing 7.5% glycerol. The number of cells/ml. was adjusted to 1.2×10^6 and the suspensions were dispensed in the containers which were then sealed. They were stored at room temperature (26 - 35°C.). Cells were counted after 24 hours storage and cultures were prepared from the stored cells to determine the number of viable cells containing viable schizonts. This was repeated every 24 hours until there were no detectable viable cells.

The results are given in Table 9 and Fig. 2. Infected lymphoid cells in the presence of 7.5% glycerol could not be recovered even after 24 hours storage. Only, approximately, 5% of the infected lymphoid cells which were stored without the presence of glycerol could be recovered after 24 hours storage. After 48 hours storage the cultures made from the stored cells did not show any viable cells left in the suspensions.

Experiment 3.5

Suspensions of infected lymphoid cells were prepared exactly as in Experiment 3.4. They were stored this time in an ordinary refrigerator at 4 - 8°C. Cells were counted and cultures were prepared as the previous experiment.

There was marked difference in recovery of infected lymphoid cells in the presence of 7.5% glycerol and without glycerol. In suspensions with 7.5% glycerol,

Table 9

Storage of infected lymphoid cells at ambient temperature (26-35°C.) and 4-8°C. in the presence or absence of glycerol (7.5%).

Duration of storage (hours)	Group	Glycerol in medium (7.5%)	Temperature of storage	Cells*/ml. before storage ($\times 10^6$)	Cells*/ml. after storage ($\times 10^3$)	Tissue culture of the cells after storage: Cells*/ml. ($\times 10^3$) at: 0	24	48	72	96 (hours)
24	A	+	ambient	1.2	800	160	160	-	-	-
	B	-	"	1.2	1000	200	20	40	80	180
	A_1	+	4-8°C.	1.2	960	190	150	270	450	750
	B_1	-	"	1.2	1200	200	350	750	840	810
48	A	+	ambient	1.2	320	64	30	-	-	-
	B	-	"	1.2	330	66	40	-	-	-
	A_1	+	4-8°C.	1.2	650	130	100	110	230	450
	B_1	-	"	1.2	850	170	250	470	750	830
72	A_1	+	4-8°C.	1.2	500	100	50	70	170	360
	B_1	-	"	1.2	800	160	120	250	480	750
96	A_1	+	4-8°C.	1.2	410	80	50	-	-	-
	B_1	-	"	1.2	700	140	90	170	250	520
120	B_1	-	4-8°C.	1.2	500	100	50	100	220	470

* Intact cells as judged by microscopic examination.

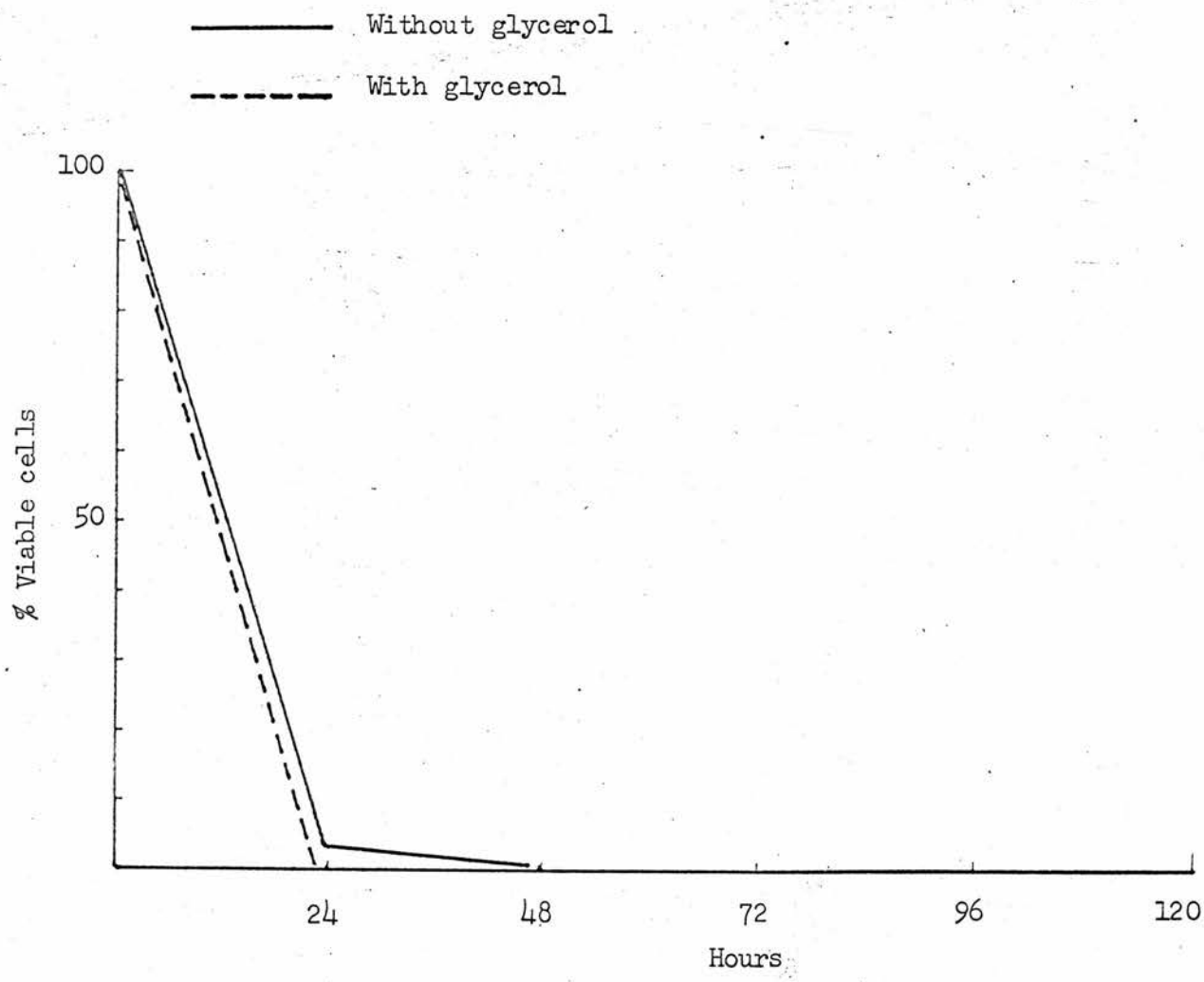

Fig. 2 Recovery of infected lymphoid cells after storage at the ambient temperatures (26-35°C.) with or without glycerol.

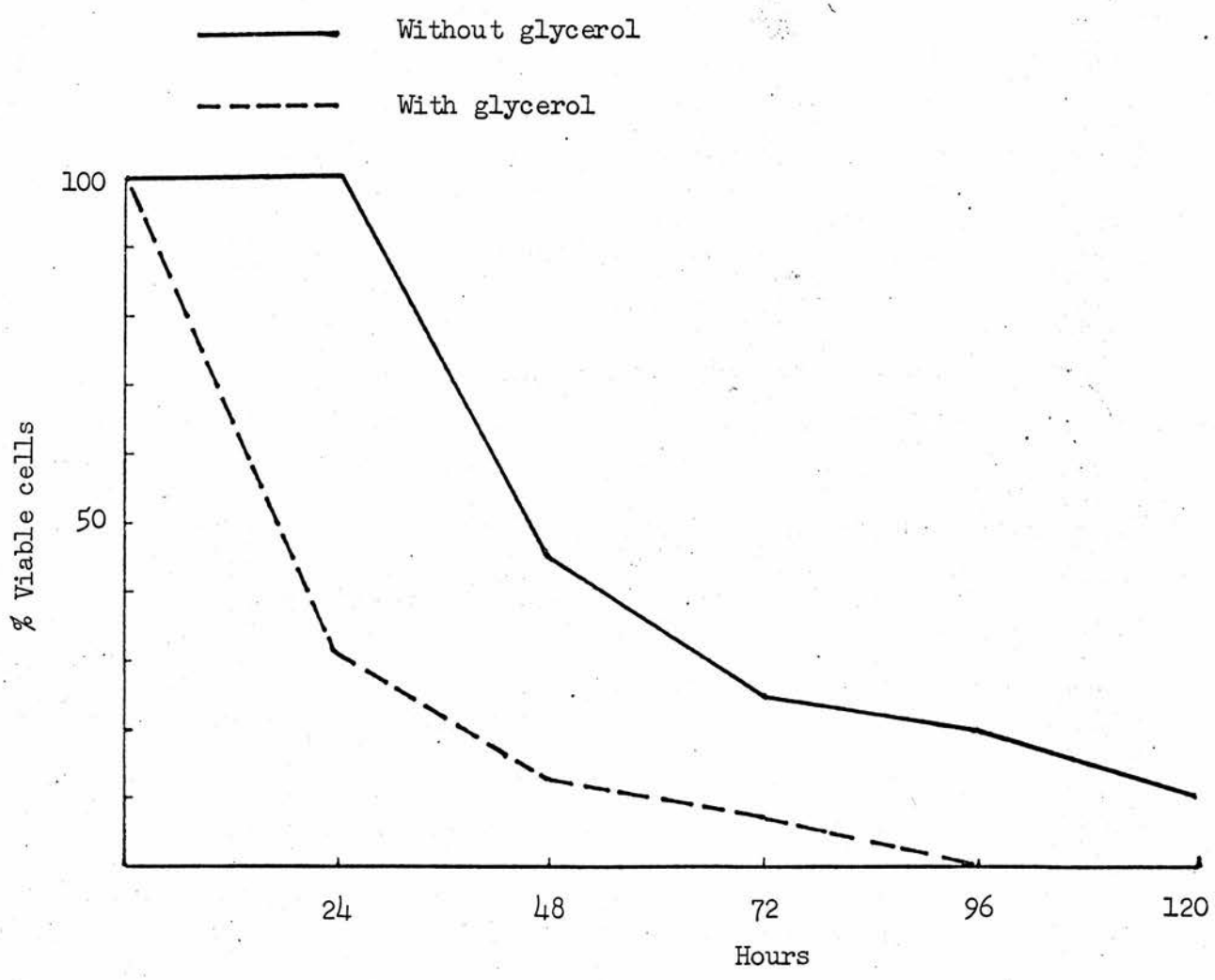

Fig. 3 Recovery of infected lymphoid cells after storage at 4-8°C. with or without glycerol.

approximately, 30% of cells were recovered after 24 hours of storage. The number of cells recovered, gradually, decreased and became too small to measure after 96 hours. Nearly 100% of the cells, in suspensions without glycerol, were viable after 24 hours but the rate of recovery decreased with the lapse of time and after 120 hours of storage only 10% of cells could be recovered (Table 9 and Fig. 2).

Experiment 3.6

Suspensions of infected lymphoid cells were prepared in the nutrient medium containing 10% glycerol as the cryoprotectant. The number of cells/ml. was adjusted to 1.2×10^6. The suspensions were dispensed in vials and were placed in a deep freeze cabinet at $-22^{\circ}C$.

The infected lymphoid cells which were preserved at this temperature were found to be no longer viable after 24 hours of storage. Replication of this experiment gave the same result.

Experiment 3.7

(a) Comparison of cryoprotectants and the methods of elution.

Suspensions of 1.2×10^6 infected lymphoid cells/ml. were prepared in the nutrient media containing 7.5%, 10% and 15% glycerol and 10% DMSO respectively. These suspensions were dispensed in vials, which were sealed, and were frozen by the slow controlled method ($1^{\circ}C$./minute) to $-70^{\circ}C$. and were stored in a Revco cabinet. Two samples were selected from each suspension at each

determination of the viability of cells. In the first sample the cryoprotectant was eluted by the slow method and in the second sample this was carried out by the rapid method. Then cells were counted and cultured.

Results are given in Table 10 and Fig. 4. The rate of recovery varied using different cryoprotectants as well as different methods of elution. Dimethyl sulphoxide showed better cryoprotection than glycerol. Glycerol at the 7.5% level showed better cryoprotection than at 10% and 15% levels. Diluting out of the cryoprotectants by the slow method considerably increased the rate of the recovery.

(b) Comparison of the effect of the rate of cooling.

Suspensions of 1.2×10^6 infected lymphoid cells/ml. were prepared in the nutrient medium containing 7.5% glycerol. These suspensions were dispensed in vials which were then sealed. Temperature was lowered in 2 ways.

(1) - Controlled slow method. The vials were immersed in a bath of 95% ethyl alcohol at 5°C. and the temperature was lowered to -30°C. at, approximately, 1°C./minute, by the addition of chipped dry ice. From this temperature to -70°C. the rate of fall in the temperature was not controlled.

(2) - Uncontrolled slow method. The vials were set in a cardboard box and were placed in a Revco deep freeze cabinet at -70°C.

The results as judged by counting, microscopic

examination and set up of cultures are given in Table 11. Controlled slow cooling did not show any advantage over the uncontrolled slow cooling method.

(c) Comparison of the effect of the equilibration period.

Suspensions of infected lymphoid cells were prepared in the nutrient media containing 7.5%, 10% glycerol and 10% DMSO respectively. They were dispensed as described in the above experiment. The cells were cooled and frozen by the uncontrolled slow method after being kept for 15, 60 and 300 minutes on the bench at 29°C. to permit equilibration to occur.

Periods for equilibration of the cryoprotectants, on both sides of cells membrane, longer than 15 minutes did not improve the rate of recovery of the infected lymphoid cells. The recovery of the cells allowed to stand for 300 minutes lapse of equilibration time was lower than that of 15 and 60 minutes periods (Table 12).

Table 10

Effects of freezing and thawing on infected lymphoid cells in the presence of different cryoprotectants and comparison of the method of elution of the cryoprotectants.

Group	Cryo-protectant	Cells*/ml. before freezing ($\times 10^6$)	Cells*/ml. after thawing ($\times 10^3$)	Method of elution of cryo-protectants	Cells*/ml. after elution ($\times 10^3$)	Tissue culture of the cells after storage: Cells*/ml. ($\times 10^3$) at: (hours) 0	24	48	72	96
A	Glycerol 15%	1.2	960	Slow	670	100	30	40	60	120
	"	1.2	"	Rapid	10	100	8	10	20	50
B	Glycerol 10%	1.2	720	Slow	630	100	70	130	230	450
	"	1.2	"	Rapid	45	100	20	30	60	180
C	Glycerol 7.5%	1.2	790	Slow	680	100	90	150	410	520
	"	1.2	"	Rapid	580	100	60	110	240	450
D	DMSO 10%	1.2	1150	Slow	920	100	100	190	450	600
	"	1.2	"	Rapid	530	100	60	90	110	200

* Intact cells as judged by microscopic examination.

Table 11

Comparison of the rate of cooling.

Group	Cells*/ml. before freezing ($\times 10^6$)	Method of freezing	Duration of storage (days)	Cells*/ml. after elution ($\times 10^3$)	Tissue culture of the cells after storage: Cells*/ml. ($\times 10^3$) at: 0	24	48	72	96 (hours)
A	1.2	1°C./min.	1	720	100	70	130	260	500
B	1.2	uncontrolled slow	1	700	100	70	120	240	460
A	1.2	1°C./min.	30	820	100	97	122	255	510
B	1.2	uncontrolled slow	30	700	100	95	112	255	502

* Intact cells as judged by microscopic examination.

Table 12

The effect of equilibration periods on freezing of infected lymphoid cells using different cryoprotectants.

Group	Cells*/ml. before freezing ($\times 10^6$)	Equilibration time (minutes)	Cryo-protectants	Cells*/ml. after elution ($\times 10^3$)	Tissue culture of the cells after storage: Cells*/ml. ($\times 10^3$) at: 0	24	48	72	96 (hours)
A	1.2	15	7.5% Glycerol	840	100	100	180	350	630
B	1.2	"	10% Glycerol	760	100	80	120	260	510
C	1.2	"	10% DMSO	900	100	90	180	360	650
A	1.2	60	7.5% Glycerol	860	100	90	150	310	540
B	1.2	"	10% Glycerol	750	100	70	140	250	460
C	1.2	"	10% DMSO	890	100	100	170	280	560
A	1.2	300	7.5% Glycerol	730	100	80	140	290	440
B	1.2	"	10% Glycerol	620	100	60	90	180	370
C	1.2	"	10% DMSO	800	100	80	140	220	490

* Intact cells as judged by microscopic examination.

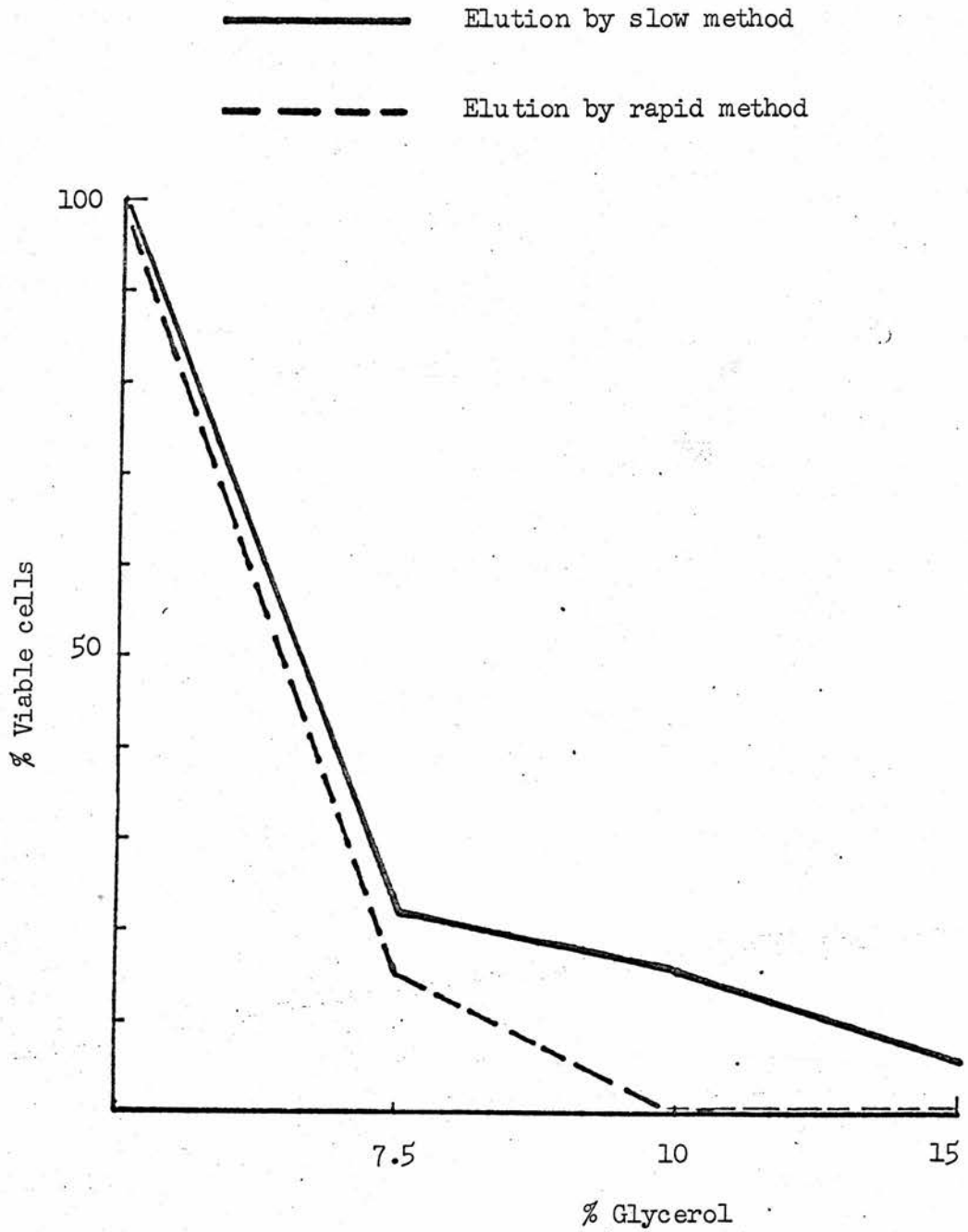

Fig. 4 Effect of the methods of elution on the recovery of infected lymphoid cells after freezing and thawing in the presence of various percentages of glycerol.

CONCLUSIONS

(i) - The addition of lactalbumin hydrolysate and yeast extract to the Eagle's medium increased the rate of the growth of infected lymphoid cells. The optimum amount of LY needed in the Eagle's medium was 40 ml. of a solution containing 25 g. lactalbumin and 5 g. yeast extract/litre.

(ii) - The optimum number of infected lymphoid cells for implantation was 10^5/ml. When this number of cells was implanted they increased by 9 fold in 96 hours, using modified Eagle's medium.

(iii) - Calf serum, as well as serum from the sheep and the horse, supported the propagation of infected lymphoid cells. Calf serum at the 10% level was as effective as when 20% was used and, therefore, the former percentage was adopted for the definitive medium.

(iv) - Strains that were grown for more than 60 passages in tissue culture were rendered agamogenous. Complete attenuation of T.annulata strains in tissue culture could be achieved, but it was not necessarily the rule.

(v) - The ambient temperature (26 - 35°C.) and -22°C. were not suitable for storage of infected lymphoid cells. Almost one hundred percent viability of infected lymphoid cells was maintained when suspensions of these cells, without the presence of glycerol, were stored at 4 - 8°C. for 24 hours.

(vi) - The slow uncontrolled rate of cooling was preferable for freezing and storing infected lymphoid cells at -70°C.

(vii) - Dimethyl sulphoxide at the 10% level was a better cryoprotectant than glycerol at the 7.5%, 10% and 15% levels, the optimum for glycerol being 7.5%. As at the time of these experiments the author had doubts on the effect of DMSO in the body of animals he decided to use 10% DMSO only for storage of the stabilates and 7.5% glycerol for storage of vaccine.

(viii) - The slow method of elution of the cryoprotectants was the method of choice.

On the basis of the information obtained from the experiments described above a vaccine was prepared. A full description of the method of the preparation of this vaccine is presented in the next chapter.

Chapter 4

PREPARATION OF VACCINE

As a result of the experiments carried out by the author (unpublished) prior to the present studies, Strain A was selected as the vaccine strain. The course of attenuation of this strain is given in Table 5. A comparison of the results of vaccination using this strain and Strain B (Table 13) and the succeeding challenge (Table 14), which were carried out at the Razi Institute, are presented here. It seems that, on the basis of the data in these tables, this selection is justifiable. This strain at the passage level used for vaccine preparation had been used by the author in many calves, and had been found safe for use in these animals.

1 - Preparation of cultures. A stabilate at the sixtieth passage was thawed and the infected lymphoid cells were implanted in a culture bottle. Five subcultures were carried out before sufficient cells were available for 100 Roux culture bottles. One hundred ml. cell suspensions (10^5 cells/ml.) were implanted into each Roux bottle. These were stoppered with sulphur free rubber bungs and were incubated at 38°C. for 4 days.

2-Harvest of infected lymphoid cells. Culture bottles were checked for any possible contamination. The contents of the bottles were poured into 250 ml. centrifuge tubes and centrifuged at 2,000 r.p.m. for 15 minutes. The supernatant fluid was discarded and

the sedimented cells were resuspended in fresh nutrient medium. The cell suspensions so prepared, were poured into the dispensing flask, which contained a magnetic stirrer bar, and the whole was placed on a magnetic mixer. The number of cells/ml. of this suspension was adjusted to, approximately, 1.5×10^6 by adding more Eagle's medium. Glycerol to 7.5% was added and the suspension was thoroughly mixed.

3 - Dispensing the vaccine. By means of a rubber tube connected to the dispensing flask the cell suspension, which was continuously stirred, was dispensed in 20 ml. vials. The vials were sealed, using rubber and aluminium caps, arranged in cardboard boxes and placed in a Revco refrigerator at $-70^{\circ}C$.

The whole procedure, from culture to dispensing, was carried out in sterilized glassware and under aseptic conditions.

4 - Control. After 24 hours, samples of the vaccine were tested for viability of the cells by setting up tissue cultures. Cultures were set up from them in ordinary bacterial, fungal and PPLO media to detect any possible contamination with these micro-organisms.

The vaccine prepared by this method had to be tested in animals and a standard method of vaccination was established prior to commencing the experiment on determination of the duration of immunity. The criteria which could be used at this stage were those measurable in

the clinical responses of animals, such as those to be used in the final challenge tests. The results obtained from a series of experiments planned for standardization of the method of vaccination will be presented in the next chapter.

Table 13

Thermal and parasitic reactions of two groups of calves which were respectively inoculated with Strain A and Strain B.

Number of animals	Strain	Mean incubation periods (days)			Mean maximum temperature (°F.)	Mean duration of pyrexia (days)	Mean erythrocytic forms (%)	Mean patent periods of schizonts (days)	
		Pyrexia	Schizonts	Erythrocytic forms				Liver	Lymph node
8	A*	13.6	13.4	0***	104.8	3.4	0	3.1	3.4
9	B**	14.6	14.7	17.4	105.8	3.1	0.9	2.8	2.8

* 290 days in tissue culture.

** 45 days in tissue culture.

*** No erythrocytic forms were produced.

Table 14

Challenge tests with Strain* No. 3 of two groups of animals, vaccinated respectively with Strains A and B.

Number of vaccinated animals	Strain	Number of control animals	Mean duration of pyrexia (days)		Number of animals showing schizonts in liver		Number of animals showing schizonts in lymph node		Number of deaths in	
			Control	Vaccinated	Control	Vaccinated	Control	Vaccinated	Control	Vaccinated
8	A	6	6.6	1.5	6	4	6	6	6	0
9	B	6	6.1	4.8	6	9	6	9	6	4

* Strain No. 3 caused mortalities of over 80% in susceptible calves.

Chapter 5

DEVELOPMENT OF DEFINITIVE METHOD OF VACCINATION

Experiment 5.1

Comparison of the reaction of animals to preserved and freshly prepared tissue culture vaccine.

The ability to store a tissue culture vaccine, for a considerable period of time without loss of potency is of great advantage.

It has previously been shown (Chapter 3) that ambient temperatures (26 - 35°C.) and -22°C. were not suitable for storage of infected lymphoid cells for any reasonable length of time. These cells could be stored for a few days at 4 - 8°C. The only method which gave reproducible results, in tissue culture, was the preservation of these cells at low temperatures (-70°C.). The aim of the present experiment was to compare the clinical and parasitological reaction of animals, inoculated with a vaccine preserved at low temperatures, with that of animals which were vaccinated with a vaccine consisting of freshly cultured schizonts of the same strain at the same passage level.

Materials

Ten calves of approximately 5 months of age were used. They were divided randomly into 2 groups each consisting of 5 calves. Repeated examination of blood smears had shown they were free from T.annulata infection. Management and tick control measures were as described in Chapter 3. Fresh culture vaccine was prepared from the infected lymphoid cells of Strain A (sixty sixth passage). These

were prepared as a suspension containing 1.5 x 10^6 cells/ml. The frozen vaccine had been prepared and preserved for a month at -70°C. Each animal received 2 ml. of one or the other type of vaccine, subcutaneously on the right side of the neck above the prescapular lymph nodes.

Observations

1 - The temperature of the calves was taken daily in the morning.

2 - Blood smears were prepared, stained and examined prior to inoculation, on the day of inoculation and from Day 10 to the end of the experiment.

3 - Needle biopsies of the right prescapular lymph nodes were carried out as described above, daily from Day 10 after vaccination. This was discontinued when 2 biopsies on successive days showed no schizonts. Schizonts were counted in the biopsy smears in relation to 1,000 lymphocytes and are shown in percentages.

4 - Enlargement of lymph nodes was checked by palpating the prescapular lymph nodes daily.

Results

Noticeable clinical reactions in both groups were the rise in temperature and enlargement of the local lymph nodes. There were no signs of depression or anorexia in the animals of either group. The prescapular lymph nodes of all the animals in both groups enlarged in size considerably, reaching a maximum at approximately the time of the onset of fever.

Thermal reactions of the animals in both groups are given in full in Appendix Table 1 and they are summarised in Table 15. Onset of fever in the calves of the group that received freshly prepared vaccine was, on average, earlier than that of the group which received the preserved vaccine. Duration of fever was also longer in the group that received freshly prepared vaccine. The maximum body temperature, although the lowest was recorded in the group that received the preserved vaccine, did not show any marked difference. Statistical analysis by the t test shows that the differences between onset and duration of pyrexia and maximum body temperatures are not significant even at the 5% level (Table 17). Reference to Table 15 shows that variations in the data obtained from the group that received the freshly prepared vaccine were greater than those of the other group.

Examination of blood smears, before and after inoculation did not reveal any development of erythrocytic forms of the parasite. The time and duration of appearance of macroschizonts are given in Table 16. They are summarised in Table 15. The prepatent period like the time to onset of fever was shorter in the group that received freshly prepared vaccine. The duration of patent period of macroschizonts was also slightly longer in the group that received freshly prepared vaccine. Application of the t test to these figures shows that they are not significantly different from each other (Table 17).

Table 15

Parasitic and thermal reactions of two groups of calves vaccinated respectively with frozen and fresh tissue culture vaccine.

Calf No.	Type of vaccine	Schizonts		Pyrexia		
		Prepatent period (days)	Patent period (days)	Day to onset	Duration (days)	Maximum (°F.)
1178	Fresh culture	11	4	11	4	104.4
1159	"	12	5	10	6	105.0
1186	"	12	3	10	4	104.4
1176	"	11	4	12	6	104.6
1182	"	13	4	13	4	104.2
Mean		11.80± 0.83	4± 0.70	11.2± 1.30	4.80± 1.09	104.52± 0.3
1165	Frozen culture	13	4	13	5	104.8
1158	"	13	4	13	4	105.0
1164	"	12	3	12	4	103.9
1135	"	13	3	14	2	103.3
1153	"	12	3	12	2	103.4
Mean		12.60± 0.54	3.40± 0.54	12.80± 0.83	3.40± 1.34	104.08± 0.78

Table 16

Percentages of macroschizonts in relation to lymphocytes in the biopsy smears from the lymph nodes of two groups of calves vaccinated respectively with frozen and fresh tissue culture vaccine.

Calf No.	Vaccine	Days related to vaccination										
		10	11	12	13	14	15	16	17	18	19	20
1178	Fresh culture	-	-	12.5	43.5	7.4	2.6	-	-	....	....	....
1159	"	-	-	-	1.0	6.5	15.3	46.5	1.0	-	-	....
1186	"	-	-	-	2.0	8.3	0.6	-	-	....	....	....
1176	"	-	-	0.1	8	4.5	0.1	-	-	....	....	....
1182	"	-	-	-	-	1.0	2.0	0.5	-	-	....	....
1165	Frozen culture	-	-	-	0.3	4.6	15.6	1.8	-	-	....	....
1158	"	-	-	-	-	6.0	10.0	16.0	1.0	-	-	....
1164	"	-	-	-	0.1	0.1	0.1	-	-	....	....	....
1135	"	-	-	-	-	0.1	1.2	0.5	-	-	....	....
1153	"	-	-	-	0.7	2.5	0.3	-	-	....	....	....

.... = Not carried out - = Negative

Table 17

Statistical analysis of thermal and parasitic reactions of two groups of calves vaccinated respectively with frozen and fresh tissue culture vaccine.

	Fresh culture		Frozen culture		D.F.	t	P
	Mean	S.D.	Mean	S.D.			
Time to onset of fever (days)	11.20	1.30	12.80	0.83	8	2.30	= 0.5
Duration of fever (days)	4.80	1.09	3.40	1.34	8	1.80	>0.1
Period before appearance of macroschizonts (days)	11.80	0.83	12.60	0.54	8	1.78	>0.1
Days of patent period (macroschizonts)	4.00	0.70	3.40	0.54	8	1.50	>0.1
Maximum body temperature (^{o}F.)	104.52	0.30	104.08	0.78	8	1.16	>0.2

Experiment 5.2

Comparison of reactions to the tissue culture vaccine in relation to the size of dose.

The number of schizonts used in the previous experiment was arbitrary and, from experience, was sufficient to cause reactions such that immunity might be expected. The time from inoculation to onset of fever in calves receiving one dose of the preserved vaccine containing 3×10^6 schizonts was from 12 to 13 days. This approximates to the usually observed incubation period in artificially induced T.annulata infection. This experiment was designed in order to study the effect of the size of the vaccinal dose.

Materials

Vaccine prepared and preserved at low temperatures (Chapter 4) and fresh vaccine from the same strain were used. Seven groups of calves were used. Three groups of 4 each were vaccinated with vaccine which had been preserved at low temperatures. The numbers of schizonts injected into animals in each group were 1.5×10^6, 3×10^6 and 6×10^6 respectively. These inoculations were all subcutaneous on the right side of the neck. Three groups of 2 calves each received fresh vaccine in doses of 1.5×10^6, 15×10^6 and 150×10^6 subcutaneously on the right side of the neck. The final group, of 2 calves, each received 150×10^6 schizonts intravenously.

Observations

1 - Rectal temperatures of the calves were taken daily in

the morning.

2 - Blood smears were prepared, stained and examined, as described above, on the day of inoculation and from Day 10 after vaccination to the end of the experiment.

3 - Lymph node biopsies, as described above, were carried out on right and left prescapular lymph nodes of animals in all the groups. This commenced in the groups which received the preserved vaccine from Day 10 after vaccination and in the groups that received freshly prepared vaccine from Day 5 after vaccination.

4 - Liver biopsies, as described above, were carried out on the animals that received freshly prepared vaccine.

5 - Prescapular lymph nodes were palpated daily for the detection of any enlargement.

6 - Packed cell volume, as described above, was estimated daily in the animals which received the freshly prepared vaccine.

Results

Clinical reactions were manifested as rise in body temperature and enlargement of the local lymph nodes.

The thermal reactions of the animals are given in full in Appendix Table 2. These are also summarised in Tables 18 and 19. Reference to these tables indicates that increase in the number of the schizonts in the inoculum reduced the prepyrexial period. Fig. 5 shows the linear regression line drawn for this correlation. Statistical analysis shows that the coefficient of correlation r is

highly significient. The data in Table 19 for the group of calves which received various doses of the freshly prepared vaccine, show that the prepyrexial period was also decreased in some animals to as little as 8 days. The duration of pyrexia does not seem to be related in any way to the doses at low levels, but when the data obtained for all the groups (Tables 18 and 19) are analysed simultaneously it appears that the duration of pyrexia was directly related to the number of the schizonts in the inoculum viz. the mean pyrexial period in the group that received 1.5×10^6 schizonts was 3.75 days and the corresponding figure in the group which received 150×10^6 schizonts was 5.5 days. The magnitude of the thermal reactions does not appear to have been related to the size of the doses or the route of inoculation.

Examination of blood smears of all the calves failed to reveal the development of erythrocytic forms. The times to appearance and duration of potency of schizonts in the local lymph nodes are summarised in Tables 18 and 19. Table 20 records the detection of macroschizonts in the lymph nodes and the liver. Reference to these tables indicates that the prepatent period was reduced as the number of schizonts in the inoculum increased. Fig. 6 shows that there was an inverse relationship between the size of the dose and the prepatent period. It also indicates that the coefficient of correlation r is highly significant. The prepatent periods in the groups which received the freshly prepared vaccine showed a similar relationship.

In one of 2 calves of the group which received 15 x 10^6 schizonts, schizonts were first detected on Day 6 but they could not be seen in the biopsy smears in the subsequent days until Day 10. The prepatent period in this group is considered to be 10 days. The patent period for macroschizonts in all the groups, without being statistically analysed, appears to be directly related to the size of the dose, viz. the mean patent period in the group which received 1.5 x 10^6 schizonts in the preserved vaccine was 3.5 days whereas the corresponding figure in the group which received 150 x 10^6 schizonts was 5 days.

Reference to Table 20 shows that in those animals which received vaccination subcutaneously the schizonts appeared in the local lymph nodes and in the liver. They did not appear in the prescapular lymph nodes on the side opposite to the inoculation. In all these animals schizonts were detected in the local lymph node approximately 24 hours before they appeared in the liver. In Group 7, in which the high dose was given intravenously, schizonts appeared in the prescapular lymph nodes of both sides and in the liver. In this group the first appearance of schizonts was in the liver and then some 48 hours later in lymph nodes of both sides. In all those animals receiving vaccine subcutaneously the local lymph nodes enlarged and the ones on the opposite side did not. In those which received the vaccine intravenously the lymph nodes did not become noticeably enlarged.

The indication from Table 21 and Figs. 7 and 8 is that anaemia, as measured by packed cell volume, was insignificant in those groups receiving 1.5×10^6 and 15×10^6 schizonts subcutaneously; a slight lowering of packed cell volume in the group receiving 150×10^6 schizonts subcutaneously is indicated. In the final group receiving 150×10^6 schizonts intravenously, however, there was a marked fall in packed cell volume in both animals and one of them died on Day 16.

The postmortem examination carried out on Calf No. 1269 showed that the carcase was emaciated. The abdominal cavity contained serous fluid, the muscles were pale and the serous membranes were yellowish in colour. The rumen contained some food and showed no pathological changes. The abomasum contained some food but no characteristic necrotic ulcers were seen nor was there any congestion. The omasum was not hard to the touch and the contents were normal. The intestines were congested, but the mesenteric lymph nodes showed slight enlargement and were seen to be congested when cut. The fatty tissues were yellow and gelatinous. The bladder contained clear urine with no sign of haemoglobinuria. Pin point petechiae, similar to those seen in tropical theileriosis, were observed on the heart. Microscopical examination of smears prepared from the liver, spleen, lymph nodes and blood revealed the presence of macroschizonts in these organs but neither erythrocytic forms nor schizonts could be detected in blood smears.

Table 18

Parasitic and thermal reactions of three groups of calves vaccinated with various doses of frozen tissue culture vaccine.

Calf No.	Number of schizonts inoculated (x 10^6)	Schizonts		Pyrexia		Maximum temperature $^oF.$
		Prepatent period (days)	Patent period (days)	Day of onset	Duration (days)	
1082	1.5	16	1	16	2	103.8
1078	1.5	19	5	18	6	106.2
1170	1.5	17	2	17	2	104.0
1154	1.5	14	6	16	5	105.1
Mean		16.5 ± 2.08	3.5 ± 2.38	16.75 ± 0.95	3.75 ± 2.06	104.77 ± 1.10
1184	3	13	3	12	4	104.4
1144	3	12	4	12	4	104.2
1024	3	14	2	13	3	104.8
1160	3	12	2	12	3	104.0
Mean		12.75 ± 0.95	2.75 ± 0.95	12.25 ± 0.50	3.5 ± 0.57	104.35 ± 0.34
1114	6	10	4	10	2	104.0
1152	6	11	3	11	3	104.2
1168	6	11	1	11	2	103.2
1046	6	10	3	10	4	105.6
Mean		10.5 ± 0.57	2.75 ± 1.25	10.5 ± 0.57	2.75 ± 0.95	104.25 ± 0.99

Table 19

Parasitic and thermal reactions of 4 groups of calves vaccinated with various doses of fresh tissue culture vaccine.

Calf No.	Number of Schizonts inoculated (x 10^6)	Route of inoculation	Macroschizonts		Pyrexia		Maximum temperature °F.	Death
			Prepatent period (days)	Patent period (days)	Day of onset	Duration (days)		
1252	1.5	s/c	12	3	13	4	104.2	-
1260	1.5	s/c	12	4	14	4	104.6	-
Mean			12	3.4	13.5	4	104.4	
1240	15	s/c	10	2	10	4	105.8	-
1248	15	s/c	10*	6	10	6	105.4	-
Mean			10	4	10	5	105.6	
1227	150	s/c	9	4	8	5	106.7	-
1229	150	s/c	8	6	8	6	106.6	-
Mean			8.5	5	8	5.5	106.65	
1269**	150	i/v	8	8	8	8	106.8	+
1253	150	i/v	8	5	8	5	106.2	-
Mean			8	6.5	8	6.5	106.5	

* A few schizonts were detected on the sixth day
s/c = Subcutaneous i/v = Intravenous.

** Died on the sixteenth day after vaccination.

Table 20

Appearance of macroschizonts of the vaccine strain in the liver and lymph node in 4 groups of animals which were vaccinated with various doses of fresh tissue culture vaccine.

Calf No.	Number of Schizonts ($\times 10^6$)	Route of inoculation	Prescapular lymph node		Liver
			Right	Left	
1252 *	1.5	s/c	+	-	+
1260 *	1.5	s/c	+	-	+
1240 *	15	s/c	+	-	+
1248 *	15	s/c	+	-	+
1227 *	150	s/c	+	-	+
1229 *	150	s/c	+	-	+
1269 **	150	i/v	+	+	+
1253 **	150	i/v	+	+	+

s/c = Subcutaneous

i/v = Intravenous

* = Schizonts first detected in lymph node

** = Schizonts first detected in liver

\- = Negative

Table 21

PCV of calves vaccinated with various doses of fresh tissue culture vaccine.

Calf No.	Number of Schizonts	Route of inoculation	Days related to vaccination																				
			-1	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1252	1.5×10^6	s/c	35	35	34	35	36	35	35	35	33	32	32	33	33	35	34	33	31	31	32	33	34	35
1260	1.5×10^6	s/c	34	34	34	35	33	32	33	34	34	34	33	34	32	31	31	32	33	34	35	34	35	35
1240	15×10^6	s/c	34	40	41	41	41	39	39	39	40	39	36	35	35	34	35	35	36	36	36	36	39	39
1248	15×10^6	s/c	35	36	36	35	37	36	34	34	37	36	36	35	34	34	33	33	34	34	36	36	35	36
1227	150×10^6	s/c	35	35	35	35	32	31	32	33	33	31	32	32	28	26	28	30	31	34	35	35	35	35
1229	150×10^6	s/c	34	34	34	35	32	31	32	31	31	30	28	28	26	26	28	28	30	34	34	33	34	34
1269	150×10^6	i/v	30	30	31	30	30	30	26	27	26	26	26	25	23	23	21	19	17*					
1253	150×10^6	i/v	28	28	28	28	29	27	29	27	28	30	24	28	25	24	23	22	21	24	23	24	22	27

s/c = Subcutaneous i/v = Intravenous * = Died

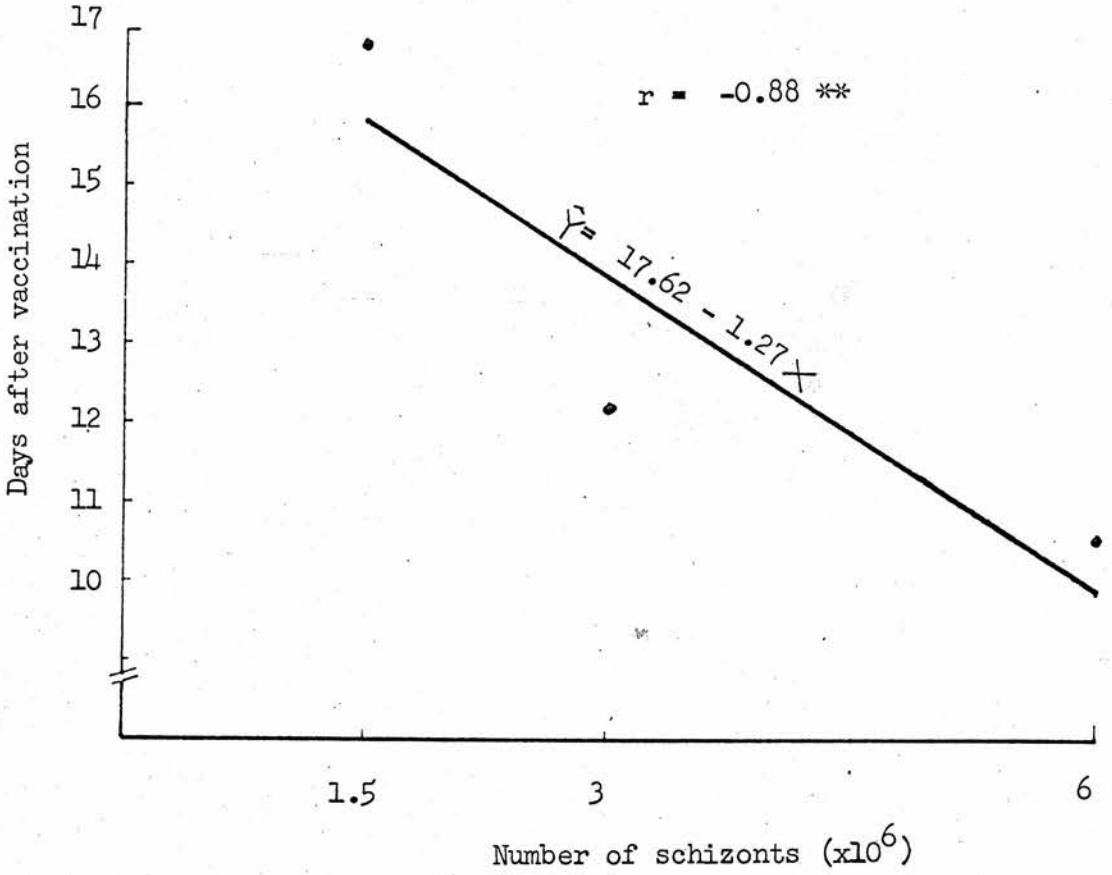

Fig. 5 Relationship of the size of dose and the mean periods to onset of fever in calves vaccinated with frozen tissue culture vaccine.

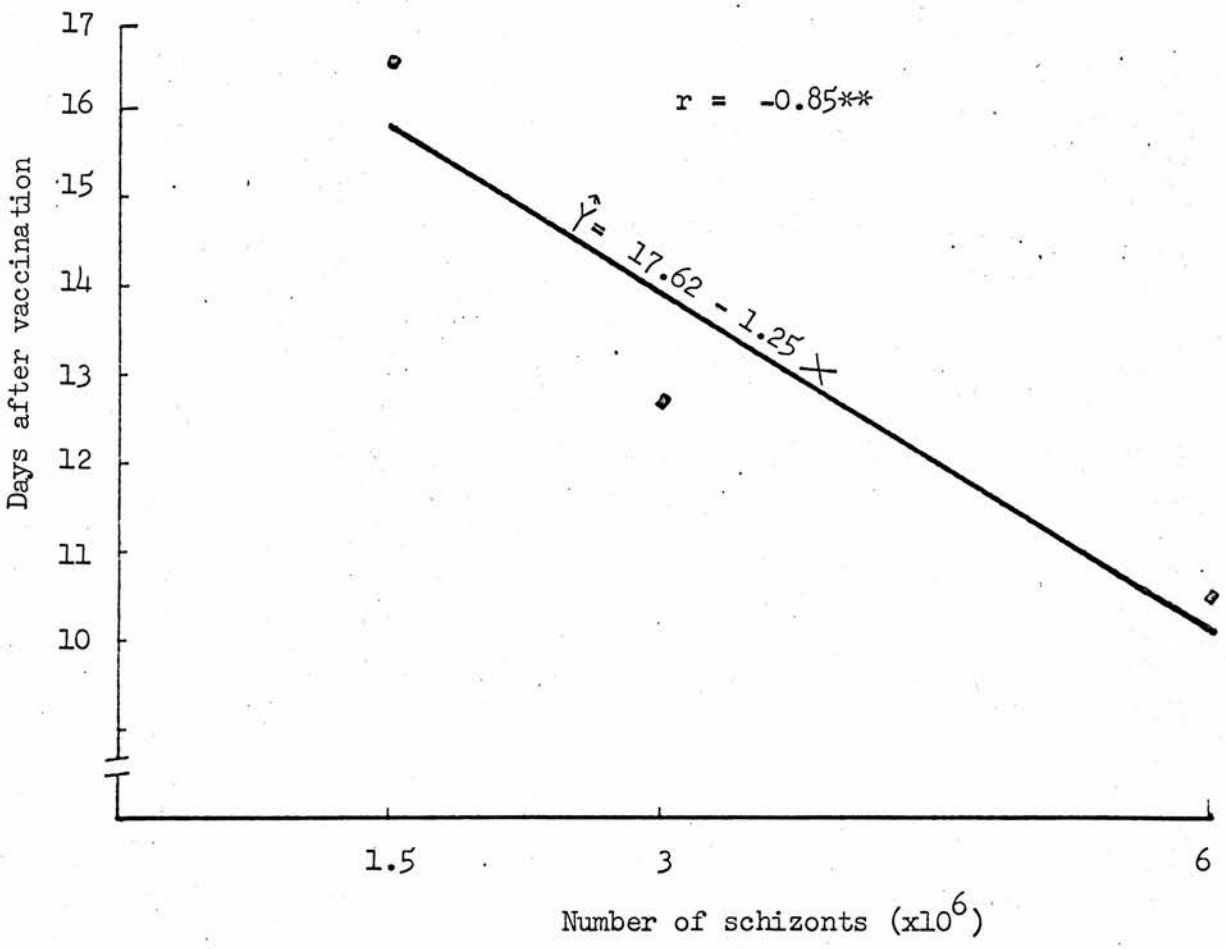

Fig. 6 Relationship of the size of dose and the mean prepatent periods in calves vaccinated with frozen tissue culture vaccine.

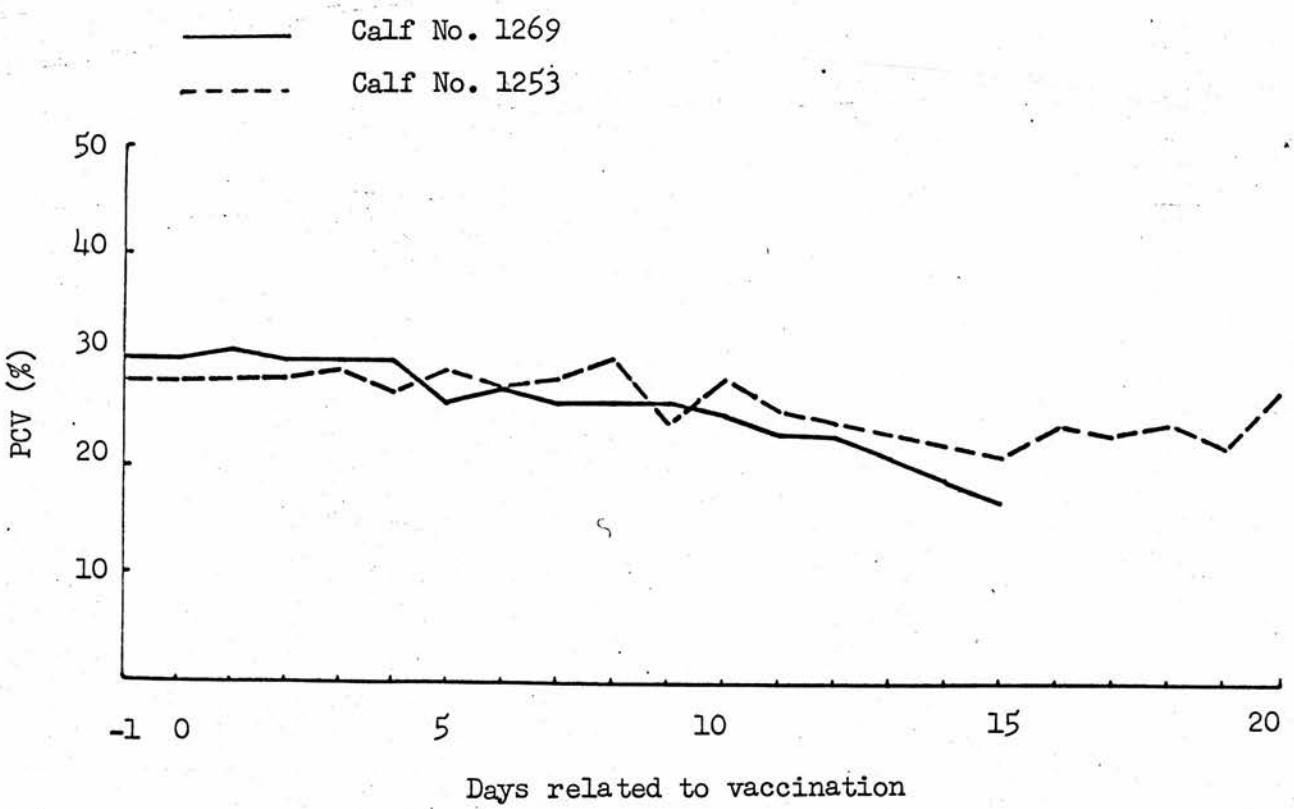

Fig. 7 Daily estimations of PCV of calves vaccinated intravenously with 150 x 10^6 schizonts of fresh tissue culture vaccine.

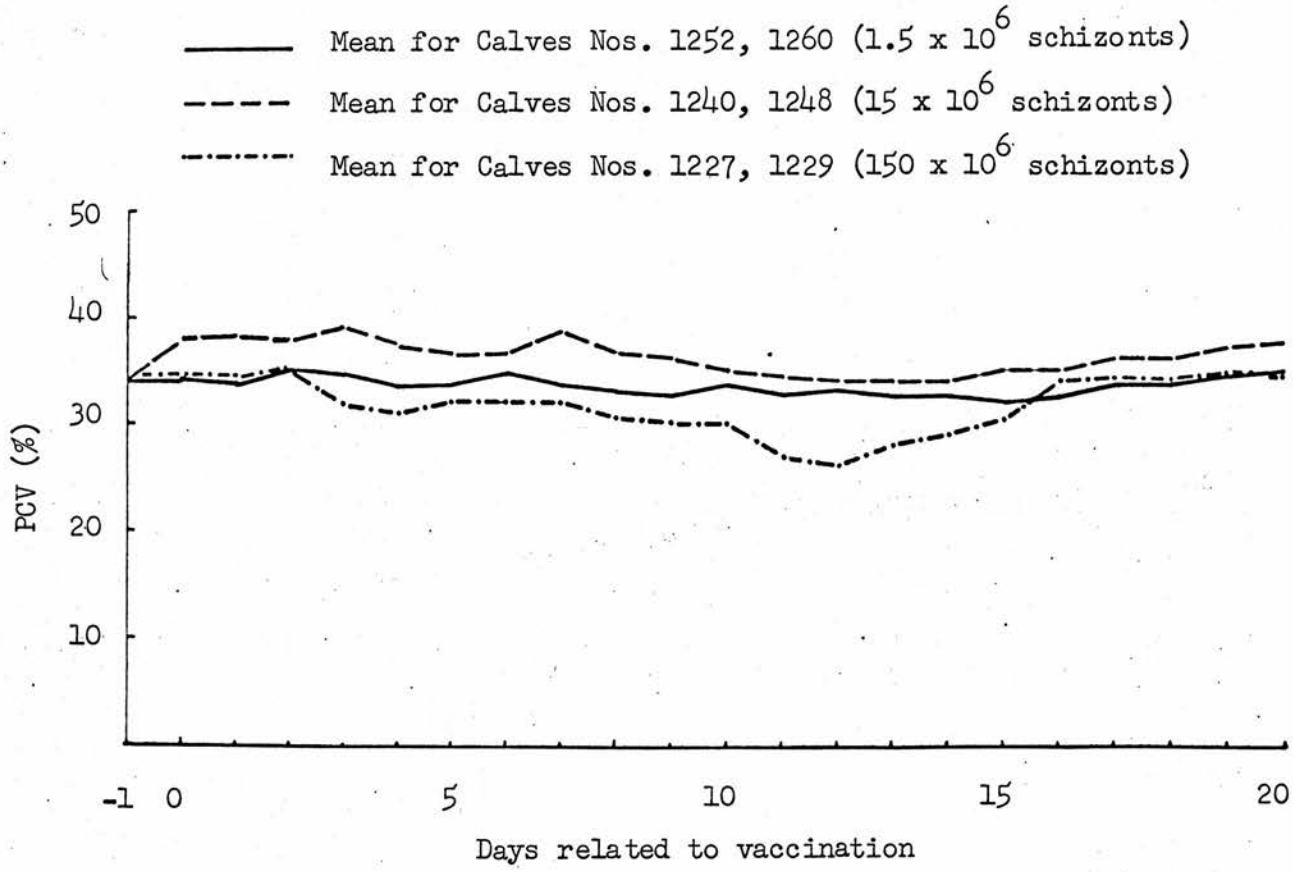

Fig. 8 Mean daily estimations of PCV of calves vaccinated subcutaneously with various doses of fresh tissue culture vaccine.

Experiment 5.3

Comparison of reactions to the tissue culture vaccine administered subcutaneously and intramuscularly.

The results of the previous vaccinations of animals, which were inoculated subcutaneously, were often variable as regards prepatent periods, patent periods and the degree of the thermal reaction. An advantage of using the subcutaneous route was that enlargement of the local lymph nodes provided a means that could be used in the field for the detection of a positive reaction to the vaccine. This experiment was designed in order to study the reactions of animals to vaccination by the intramuscular route and to compare the results with those when the subcutaneous route was used. The intravenous route was not used because as was seen in the previous experiment it could cause a generalised infection and, besides, it was not a practical route for administration of vaccine in the field.

Materials

Twenty-four Friesian calves of approximately 4 months of age were used. These calves were divided into 3 groups of 8. They were inoculated on the right side of the neck with the vaccine which had been preserved at low temperatures. The first group received 3×10^6 schizonts subcutaneously, the second group received 3×10^6 schizonts intramuscularly and the third group received 1.5×10^6 schizonts intramuscularly. The management and tick control measures were as described in Chapter 3.

Observations

1 - Rectal body temperatures of the calves were taken daily in the morning, commencing from the day before inoculation.

2 - Biopsies of the lymph node, as described above, were carried out on both prescapular lymph nodes daily from the first day of fever until the temperature returned to normal.

3 - One liver biopsy was carried out as described above on each animal in the second and third groups only. It was taken at the time the thermal reactions rose to 104°F. or above.

4 - The local lymph nodes were palpated daily and any enlargement was recorded.

5 - Blood smears were prepared from the day before inoculation and then daily as described above.

6 - Blood for preparation of sera was withdrawn from the calves by jugular puncture on the day prior to inoculation and subsequently on Day 30. The sera were preserved at -22°C. until used.

7 - The indirect immuno-fluorescent antibody test, using a tissue culture antigen (Strain A schizonts) and a fluorescein isothiocyanate conjugated gamma globulin of rabbit antibovine gamma globulin, was used for detection of circulating antibodies and the determination of the titre.

Results

Thermal reactions of all the calves are summarised in

Table 22. Reference to this Table shows that the differences between the first group, receiving 3 x 10^6 schizonts subcutaneously, and the second group, receiving 3 x 10^6 schizonts intramuscularly, were for practical purposes very small. These differences were in the time before onset of fever and duration of pyrexia viz. in the first group the mean time to onset of fever and the mean duration of pyrexia were 13.75 and 3 days respectively. The corresponding figures for the second group were 12.35 and 3.12 days. The results from the third group which received 1.5 x 10^6 schizonts intramuscularly differ from the other 2 groups viz. the corresponding figures in this group are 15 and 2.5 days. Table 22 also shows that the results in the second group are more uniform than those in the first and the third groups. In the third group noticeable variations in the duration of pyrexia and the degree of fever can be seen. The lowest, 103.4°F., and the highest 107.2°F., were recorded in this group. The lowest duration of pyrexia, one day, was also in this group.

The changes in the size of the local lymph nodes are presented in Table 23. All the local lymph nodes in the first group, as was expected, enlarged substantially. Enlargement of the local lymph nodes in the second and the third groups, which received the vaccine intramuscularly, was irregular and of lower degree.

The examination of blood smears in all the groups revealed no erythrocytic forms of the parasite. Reference to Table 23 shows that schizonts were detected in all the

local lymph nodes of the calves in the first group. The presence of schizonts in the prescapular lymph nodes of the side opposite to that of inoculation could not be established in this group. In the second and the third groups schizonts were first detected in the liver. All liver biopsies with one exception showed the presence of schizonts. The exception, Calf No. 1356, underwent a very minor thermal reaction showing no schizonts in either liver or lymph nodes. The intramuscular inoculation produced schizonts in the opposite prescapular lymph nodes in a few animals late in the reaction and in some cases no parasites appeared in the lymph node of the side of injection. In all cases where schizonts were found in the lymph nodes of the left side they were found in that of the right but the reverse did not apply.

Tables 24 and 25 present the results of the indirect fluorescent antibody test on sera from the calves in this experiment. The inoculation by the intramuscular route even when the dose was no more than 1.5×10^6 schizonts produced titres as high as and in some cases higher than were produced by subcutaneous inoculation. The highest titre obtained, 1:512, was in the animal which received 1.5×10^6 schizonts intramuscularly. In this group one animal failed to produce a titre of 1:4 and another produced a titre at this level only.

Table 22

Thermal reactions of three groups of calves vaccinated with frozen tissue culture vaccine.

Group No.	Number of schizonts inoculated	Route of inoculation	Animal No.	Pyrexia		
				Days to onset	Duration (days)	Maximum (oF)
1	3×10^6	s/c	1345	13	3	104.8
			1341	14	3	104.8
			1378	14	3	105.6
			1349	13	4	104.8
			1374	14	3	104.8
			1351	15	3	104.6
			1234	14	2	104.2
			1284	13	3	104.0
			Mean	13.75 ± 0.7	3 ± 0.53	104.7 ± 0.47
2	3×10^6	i/m	1366	11	4	105.0
			1353	13	3	104.4
			1368	13	3	105.0
			1372	11	3	104.6
			1360	13	3	104.8
			1355	13	3	105.4
			1385	12	3	104.2
			1339	13	3	104.2
			Mean	12.37 ± 0.91	3.12 ± 0.35	104.7 ± 0.42
3	1.5×10^6	i/m	1355	16	2	106.0
			1337	14	4	107.2
			1318	14	4	106.0
			1286	16	2	106.0
			1343	14	4	105.4
			1356	15	1	103.4
			100	17	1	104.0
			1354	14	2	103.4
			Mean	15 ± 1.19	2.5 ± 1.35	105.17 ± 1.40

Table 23

Parasitosis and lymph node hyperplasia in three groups of calves vaccinated with frozen tissue culture vaccine.

Group No.	Number of Schizonts inoculated	Route of inoculation	Calf No.	Size of local lymph node	Schizonts		
					Liver	prescapular lymph node	
						Left	Right
1	3×10^6	s/c	1345	Enlarged	....	-	+
			1341	"	....	-	+
			1378	"	....	-	+
			1349	"	....	-	+
			1374	"	....	-	+
			1351	"	....	-	+
			1234	"	....	-	+
			1284	"	....	-	+
2	3×10^6	i/m	1366	S.Enlarged	+	+	+
			1353	N.Enlarged	+	-	-
			1368	"	+	-	-
			1372	"	+	-	-
			1360	S.Enlarged	+	-	+
			1335	N.Enlarged	+	-	-
			1385	S.Enlarged	+	-	+
			1339	N.Enlarged	+	-	-
3	1.5×10^6	i/m	1355	N.Enlarged	+	-	-
			1337	S.Enlarged	+	+	+
			1318	"	+	+	+
			1286	N.Enlarged	+	-	-
			1343	S.Enlarged	+	-	+
			1356	N.Enlarged	-	-	-
			100	"	+	-	-
			1354	"	+	-	-

s/c = Subcutaneous
i/m = Intramuscular
.... = Not carried out
S. = Slightly
N. = Not
+ = Schizonts were detected
- = Schizonts were not detected

In Groups 2 and 3: Schizonts in all the cases were first detected in the liver.

Table 24

Indirect fluorescent antibody titre of sera from three groups of calves vaccinated with frozen tissue culture vaccine.

Group No.	Number of Schizonts inoculated	Route of inoculation	Calf No.	Titre							
				1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512
1	3×10^6	s/c	1345			+					
			1341				+				
			1378				+				
			1349					+			
			1374			+					
			1351			+					
			1234		+						
			1284			+					
2	3×10^6	i/m	1366							+	
			1353						+		
			1368					+			
			1372					+			
			1360						+		
			1335					+			
			1385							+	
			1339				+				
3	1.5×10^6	i/m	1355				+				
			1337								+
			1318						+		
			1286					+			
			1343						+		
			1356*								
			100		+						
			1354	+							

s/c = Subcutaneous

i/m = Intramuscular

* Did not show positive reaction

Table 25

Comparison of indirect fluorescent antibody titre of sera from 3 groups of calves vaccinated with frozen tissue culture vaccine.

Group No.	Number of Schizonts inoculated (x 10^6)	Route of inoculation	Positive reactions			Positive reaction/ calves inoculated
			Titre	Number of calves	%	
1	3	s/c	64	1	12.5	8/8
			32	3	37.5	
			16	4	50.0	
2	3	i/m	256	2	25.0	8/8
			128	2	25.0	
			64	3	37.5	
			32	1	12.5	
3	1.5	i/m	512	1	12.5	7/8
			128	2	25.0	
			64	1	12.5	
			32	1	12.5	
			8	1	12.5	
			4	2	25.0	

s/c = Subcutaneously

i/m = Intramuscularly

Titles are in reciprocal

Experiment 5.4

Comparison of reactions to the tissue culture vaccine in relation to the age of the animal.

So far the animals used were under one year of age, the majority being about 6 months old. It was intended, finally, to extend the vaccination to adult cattle. According to the experience of the present author (unpublished work) when similar vaccine had been used on a farm with 150 cows, 70 calves and 80 heifers, all of Friesian breed, 4 cows showed very severe reactions and 3 died of theileriosis, a mortality rate of 2%, while no mortality occurred in heifers or calves. This experiment was designed to study the relation between the age of the animal and the degree of reaction to the T.annulata tissue culture vaccine. Confirmation of previous experience would contraindicate use of the vaccine in adult cattle, and alternatives would have to be sought.

Materials

Fifteen Friesian calves of 7 to 9 months of age (considered as the first group) and 15 female Friesian cattle of 16 to 18 months of age (considered as the second group) were used. The animals were maintained under the same conditions. The management and tick control measures were as described above. Vaccine of the same batch as used above and which had been preserved at -70°C. was used. Both the older and younger animals received the same dose (3×10^6 schizonts). The vaccine was inoculated

subcutaneously on the right side of the neck, above the prescapular lymph node.

Observations

1 - Rectal temperatures were recorded daily in the morning.

2 - Blood smears were prepared, stained and examined as described above, the day before inoculation and daily from Day 10 after vaccination.

3 - Biopsies from the lymph node on the side of inoculation were carried out on all the animals daily from Day 10 after vaccination until schizonts disappeared from the biopsies on 2 successive days. Thin smears were prepared from the biopsy material and stained with Giemsa stain.

4 - Lymph nodes on the side of inoculation and the opposite side were palpated daily. They were measured along the longitudinal axis prior to inoculation and on Day 17 after vaccination. The first measurement could not be regarded as accurate in the older animals because of the presence of fat tissue around the lymph nodes. In younger animals, the nodes were more easily discernible and, therefore, more easily measured.

Results

Thermal reactions of the first group, 7 to 9 months old, and the second group, 16 to 18 months old, are presented in full in Appendix Table 4. A chart of the mean daily body temperatures for the first group and the

second group is given in Figs. 9 and 10. The percentages of animals, of the first and second groups, showing onset of fever, when 103°F. or above is considered as the commencement of fever, on certain days after vaccination are demonstrated in Fig. 11, which also shows the corresponding figures for these two groups, when elevation of the body temperature to 104°F. or above is considered as the onset of fever. Fig. 12 similarly shows a comparison of the duration of fever using similar criteria in the same manner as in Fig. 11. Table 26 shows the summary of the thermal reactions in the first and the second groups. Reference to these tables and Fig. 7 indicates that the thermal reactions in the second group, in which the animals were older, were greater both in magnitude and duration than in the first group. The difference was more outstanding when the elevation of the body temperature to 104°F. or above is considered. In this respect 2 calves in the first group did not attain this temperature and they are not presented in the mean. In no case did pyrexia last for longer than 3 days. In the second group only one animal failed to show a temperature of 104°F. or above and periods of pyrexia as long as 6 days were recorded.

There was a difference in increase in size of the local lymph nodes in the first and the second groups. Table 27 gives the measurements of the lymph nodes in the animals of each group on 2 occasions; on the day before inoculation and on Day 17 after vaccination. It also

shows the mean and the difference between the means. The application of the Student t test to these means shows that the enlargement of the lymph nodes of the animals in the second group was significantly greater than that of those in the first group. **

Examination of blood smears before and during the course of the experiment showed no erythrocytic forms of the parasite in either of the 2 groups. Table 27 is a summary of the prepatent periods and the days of patency of macroschizonts in the local lymph nodes of both groups. Reference to this table indicates that the mean prepatent period in the first group was shorter than that in the second group, but the mean patent period was longer in the second group than in the first group.

** $t = 3.23$

$D.F. = 28$

$P \leq 0.01$

Table 26

Thermal reactions of two groups of animals vaccinated with frozen tissue culture vaccine.

	Animal No.	Fever to 103°F or above			Fever to 104°F or above	
		Day of onset	Duration (days)	Maximum (oF.)	Day of onset	Duration (days)
Group 1 (calves)	739	12	2	104.2	13	1
	763	14	4	105.8	14	3
	755	15	1	104.0	15	1
	767	12	3	105.3	13	2
	765	12	6	105.0	14	2
	745	12	3	104.8	13	2
	743	13	2	103.2	-	-
	757	14	5	105.0	15	3
	761	12	2	104.0	13	1
	753	14	4	105.0	14	1
	741	12	4	105.6	13	3
	749	13	5	104.6	14	2
	729	13	5	105.4	14	3
	751	12	6	105.2	13	2
	747	14	2	103.2	-	-
	Mean	12.93	3.6	104.6	13.69*	2*
Group 2 (heifers)	1092	14	7	105.6	14	6
	1072	14	6	106.6	15	4
	1026	17	2	103.4	-	-
	1032	16	6	105.0	19	3
	1070	14	5	105.4	14	4
	1010	15	5	106.0	15	3
	1086	13	7	106.0	13	4
	1014	15	2	104.0	16	1
	1102	14	5	104.6	14	2
	1050	12	7	106.4	13	5
	1068	13	8	105.8	15	5
	1074	14	4	106.4	15	3
	1004	14	7	105.6	14	6
	1018	12	8	107.2	12	6
	1030	12	5	105.2	12	5
	Mean	13.93	5.55	105.54	14.35**	4.07**

* Mean of 13 animals ** Mean of 14 animals

\- = did not show fever.

Table 27

Parasitosis and lymph node hyperplasia in two groups of animals vaccinated with frozen tissue culture vaccine.

	Animal No.	Schizonts		Size of the local lymph node in mm.		
		Prepatent period (days)	Patent period (days)	Before	After	Difference
Group 1 (calves)	739	12	4	30	100	70
	763	14	4	35	90	55
	755	14	2	35	80	55
	767	12	3	35	80	45
	765	13	4	30	110	80
	745	13	2	40	70	30
	743	13	4	45	80	35
	757	15	4	40	80	40
	761	13	3	35	70	35
	753	14	3	35	90	55
	741	12	4	35	100	65
	749	13	4	30	80	50
	729	13	4	30	110	80
	751	13	4	35	100	65
	747	14	3	35	85	45
Mean		13.2	3.46	35	87	52
Group 2 (heifers)	1092	14	7	40	160	120
	1072	14	6	35	110	75
	1026	13	1	40	80	40
	1032	17	6	45	80	35
	1070	14	5	30	110	80
	1010	15	6	40	120	80
	1086	13	5	30	100	70
	1014	15	2	45	70	25
	1102	14	4	40	160	120
	1050	13	6	40	170	130
	1068	13	8	40	140	100
	1074	14	4	45	120	75
	1004	14	7	35	160	125
	1018	12	8	35	140	105
	1030	12	8	40	160	120
Mean		14.13	5.53	38.66	125.33	86.67

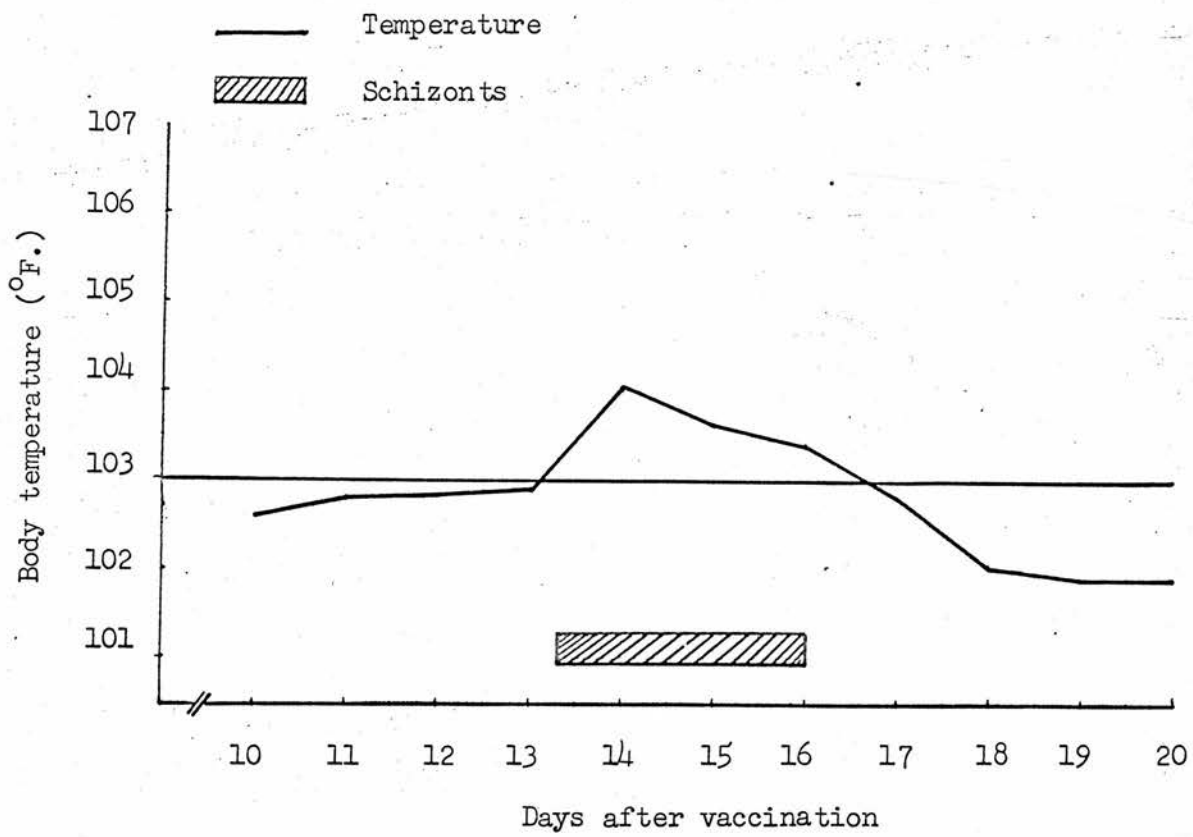

Fig. 9 Mean daily body temperatures of calves 7 to 9 months old.

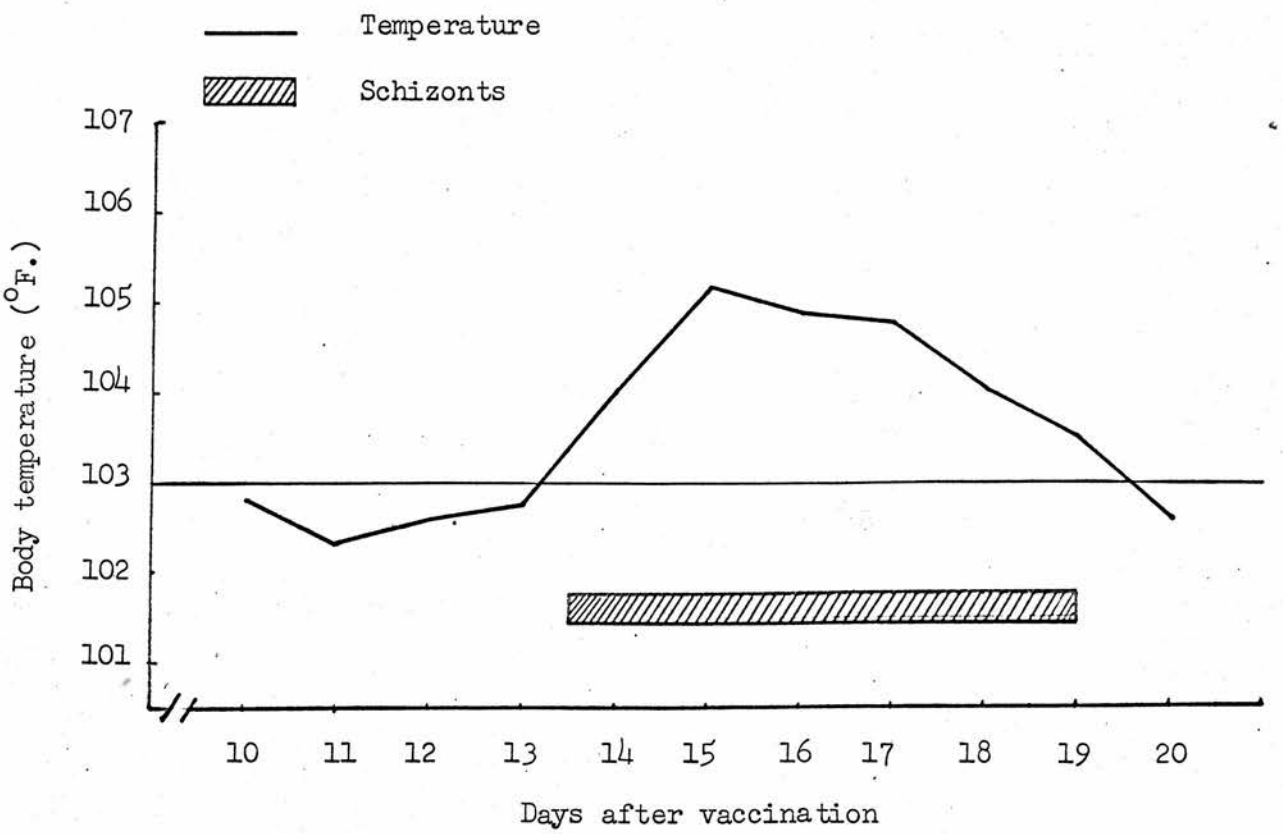

Fig. 10 Mean daily body temperatures of heifers 16 to 18 months old.

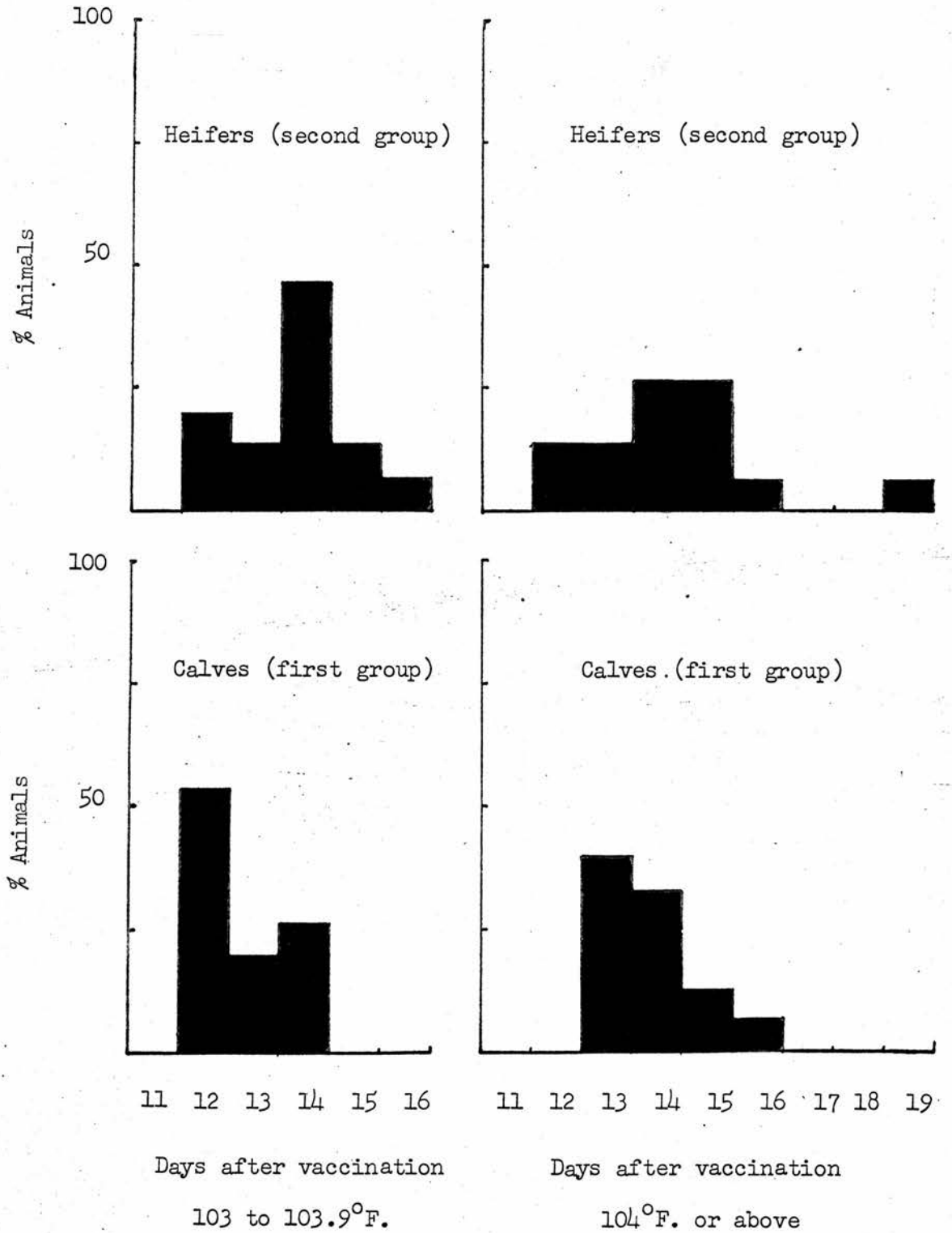

Fig. 11 Day of onset of fever (103.0 - 103.9°F. or 104°F. and above) in animals of different ages.

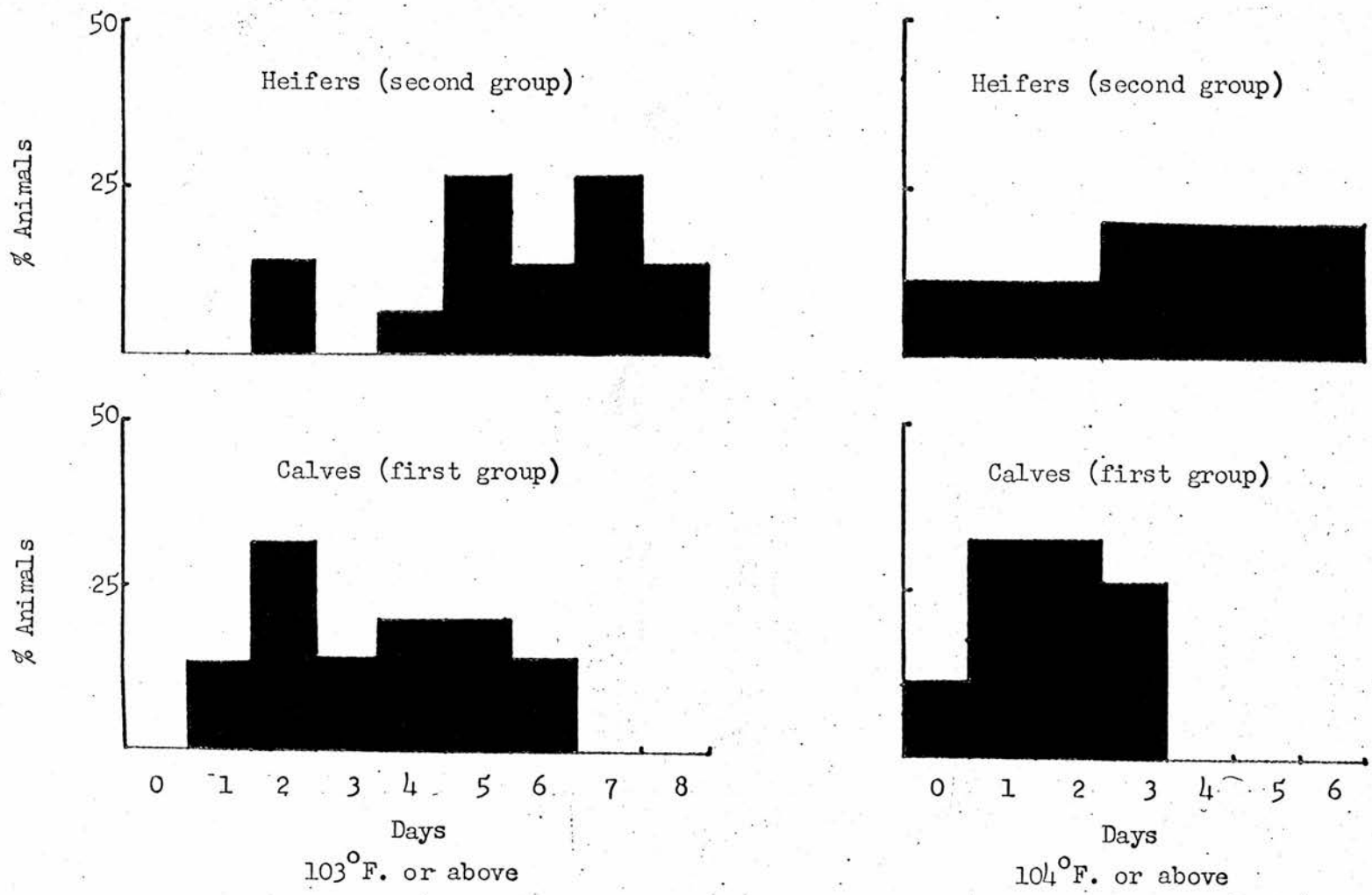

Fig. 12 Duration of febrile reactions 103°F. or above and 104°F. or above in animals of different ages.

DISCUSSION AND CONCLUSIONS

The experiments described were designed to determine a definitive method of vaccination for use in the experiments subsequently to be carried out on the immunogenic characteristics of the vaccine. Assessment had to be based mainly on the criteria of clinical reactions to the vaccine and establishment and development of the schizonts in the subject animals.

There was very little practical difference between the use of the preserved vaccine and freshly prepared vaccine. The difference in the reactions could be accounted for by the assumption that in the freshly prepared vaccine there were more viable schizonts than in that which had been frozen. The experiment on comparison of reactions to the size of dose indicated that the thermal incubation period bears an inverse relationship to the number of schizonts inoculated. Variation shown in the duration of fever in animals inoculated with the 2 types of vaccine can be attributed to individual susceptibilities. It appears that the reactions caused by the 2 vaccines were comparable in both groups and that the great advantages of being able to use a standard stabilised vaccine by freezing outweighed the disadvantages of minor differences in reactions produced by the 2 types of vaccines. One would therefore feel justified in using, for experiments designed to lead to a vaccine for field use, the stabilised frozen vaccine.

In the experiment on the relationship of reactions to

the size of dose it was shown that there are linear inverse relationships between the size of dose and the time to onset of fever and the prepatent period. Although one animal which received 150 x 10^6 schizonts intravenously died of theileriosis there was no significant relationship between the size of dose and the severity of the reactions in those that received frozen vaccine. The shortest time to the onset of fever was found to be 8 days. The shortest previously reported period following an artificial infection was 10 days (Sergent et al. 1945). It was observed that the time to onset of fever and the prepatent period, when high dosages of schizonts were inoculated, were related to dose rather than to the route of inoculation, namely intravenous or subcutaneous. In the first, second, third, fourth and fifth groups, macroschizonts became patent at first in the prescapular lymph nodes of the side of the inoculation and later in the liver. They could not be detected in the prescapular lymph nodes of the side opposite to the site of inoculation. In the sixth group, schizonts became patent at first in the liver and after some 48 hours they could be detected in the lymph nodes of both sides. These findings indicate that, when freshly cultured vaccine is used intravenously in high dosage, the schizonts eventually spread to all organs and lymph nodes but, when it is inoculated subcutaneously, they can be detected only in the liver in addition to the local lymph nodes. The results obtained in the groups which received various

dosages of freshly prepared vaccine indicate that when the schizonts of the vaccine strain are inoculated in very large numbers, they can cause anaemia. The extent of this anaemia, as judged by the estimation of PCV, was correlated with the degree of the severity of reactions. In Calf No. 1269 in which parasites generalized in the body, anaemia was more intense than in the other animals. The cause of this anaemia needs investigation, as the vaccine strain does not produce erythrocytic forms of the parasite. The presence of icterus, as revealed by postmortem examination, is possibly the result of destruction of haemoglobin in the liver.

In the experiment on the route of inoculation the intravenous route was not used as it was not considered to be practicable in the field. Comparison of the times to onset of fever in the second and the third groups, which received intramuscularly 1.5×10^6 and 3×10^6 schizonts respectively, supports the results obtained in the experiment on comparison of reactions to doses of different size; increase in the number of schizonts in the inoculum shortens the time to the onset of fever irrespective of the route of inoculation. The mean duration of fever (2.5 days) in the third group was shorter than that in the other 2 groups. There were, however, great variations within this group. The number of days of pyrexia ranged from one to 4 and in some cases the level of the body temperature attained was very high. The mean periods of fever in the first and the second groups,

which were inoculated by subcutaneous and intramuscular route respectively, showed little difference but the results from the second group were more uniform, within the group, which was an advantage.

Macroschizonts were detected in all the animals of the first, second and, except in one calf, third groups. In the second and the third groups the parasite became patent first in the liver and later in the lymph nodes. It seemed that the parasites, in the second and third groups, inoculated intramuscularly, took a route other than through the prescapular lymph nodes. Their appearances in the regional lymph nodes, possibly, and in the lymph nodes of the side opposite to inoculation, definitely, were the results of a second invasion through the circulating blood. It appears that there is a risk of generalization of the parasites in the body if the vaccine is administered intramuscularly.

Pipano, Cahana, Feller, Shabat and David (1969) used IFA for assessing the response of cattle to immunization against T.annulata, using an antigen prepared from ground liver and spleen that harboured numerous schizonts. In the present work tissue culture schizonts were used as the antigen in the IFA test for detection of response of cattle to T.annulata vaccine inoculated either subcutaneously or intramuscularly. The present author (1968 unpublished work) used tissue culture schizonts as the antigen in the direct and the indirect fluorescent antibody test but no other worker had published the use of tissue culture

schizonts in the IFA test for Theileria until Burridge and Kimber (1972) described their use in work on T.parva.

The results from the IFA test on sera obtained on the thirtieth day after vaccination showed that titres were significantly higher when the vaccine had been inoculated intramuscularly. Although 1.5×10^6 schizonts inoculated intramuscularly produced the highest titre in one calf the results were more uniform in the group that received 3×10^6 schizonts intramuscularly. Burridge (1971) stated, although a significant rise in IFA titre following experimental infection with T.parva was indicative of a degree of immunity to severe challenge, there appeared to be no correlation between the degree of immunity to challenge and the peak IFA titre. It is not yet clear whether the findings of Burridge (1971) are applicable to T.annulata infection. If it could be shown that there was some correlation between immunity and the IFA titre, then the inoculation of T.annulata tissue culture vaccine by the intramuscular route, ignoring even the risk of generalization, would be the method of choice. Until such evidence is available subcutaneous inoculation is to be preferred because of its simplicity and the fact that it almost invariably causes swelling of the local lymph node which can be detected from the fourth or fifth day after inoculation. It has the added advantages that generalization does not occur as it does after use of the intramuscular route and it is a more satisfactory method for use in the field.

In the experiment on comparison of vaccinal reaction in relation to the age of the subject animals it was observed that when elevation of temperature to 103°F. or above was considered as the onset of fever, in both groups it commenced 12 days after vaccination but the percentages of animals in each group that showed fever on this day varied. In the first group the majority of the animals (53.33%) showed fever on Day 12, whereas in the second group only 20% showed fever on this day; the majority (46.66 %) showed fever on Day 14. In general the time to onset of fever in the second group was longer than in the first. Jarret, Crighton and Pirie (1969) suggested that in T.parva infection the number of macroschizonts in the body of animal should reach a certain number before the onset of fever. If this theory can be extended to T.annulata, then it can be argued that although the number of schizonts inoculated into these 2 groups of animals was the same it took longer for the schizonts in the second group to reach the ratio of schizonts to the live body weight necessary for the onset of fever. This experiment confirmed previous experience of the author, that older animals were more susceptible to the vaccine prepared from Strain A.

Chapter 6

STUDIES ON THE DURATION OF IMMUNITY ENGENDERED IN BOVINE ANIMALS BY ADMINISTRATION OF A TISSUE CULTURE VACCINE OF T.ANNULATA

Experiments were designed to determine the immunogenic properties of the tissue culture vaccine prepared, preserved and administered on the basis of the results of the exploratory work described earlier in this thesis. Observations on the haematology of the animals at vaccination and at challenge were included as an indication of the effects of the immunogenic reactions and the response of the animals to the virulent challenge. In the challenge tests heterologous strains were used.

Experiment 6.1

Challenge test 4 months after vaccination.

Materials

Vaccination.

1 - Ten young bovine animals as shown in Table 28 were used. These were divided into 3 groups. Group 1 consisted of 3 calves of 10 to 12 months old. Groups 2 and 3 consisted of 7 calves from 4 to 5 months old.

2 - Vaccine which had been prepared and frozen as described above from Strain A was used as the immunizing agent. The dose in each animal contained 3×10^6 schizonts. Inoculations were made

subcutaneously on the right side of the neck. Groups 1 and 2 were vaccinated. The animals in Group 3 were left unvaccinated as controls.

Challenge.

1 - All the vaccinated animals and the control group were challenged 4 months after vaccination.

2 - Strain C was used as the challenge strain. Infected blood was injected into Calf No. 1143. At the peak of thermal and parasitic reactions 500 ml. blood were drawn from this calf into a beaker containing sodium citrate, as anticoagulant. The infected citrated blood so prepared was used as the challenge inoculum. Each calf received 40 ml. of this blood subcutaneously on the left side of the neck, the side opposite to the site of vaccination. Previous experience had shown that 5 ml. of virulent infected blood were sufficient to produce a fatal theileriosis due to T.annulata, therefore, 8 times this amount was considered to be adequate for infection.

Observations

Vaccination.

1 - Body temperature was taken daily in the morning.

2 - Blood smears of the calves were prepared, stained and examined daily as described above.

3 - The local prescapular lymph nodes and those on the side opposite to the site of inoculation were palpated daily to detect any enlargement.

4 - Red cell counts, white cell counts, estimation of

packed cell volume and haemoglobin, as described above, were carried out the day before vaccination, on the fourth day after vaccination and thereafter every fourth day.

5 - From the tenth day after vaccination needle biopsies of the right and the left prescapular lymph nodes were carried out. Thin smears were prepared from the lymph and stained with Giemsa stain. The biopsies were discontinued if, on 2 successive days, it was shown that schizonts had disappeared.

Challenge.

The observations in the challenge test were similar to those in the vaccination except that haematological studies were made on the day before challenge and thereafter at weekly intervals after challenge. Biopsies were restricted to the lymph nodes of the side of inoculation. Macroschizonts were counted as described in Experiment 5.1.

Results

Vaccination.

Daily body temperatures of the calves, from Day 10 to Day 20 are recorded in Appendix Table 5. The thermal reactions are summarised in Table 33. The mean time to onset of fever, the mean duration of pyrexia and the mean maximum body temperature corresponded with the results observed in the experiments described in the previous chapter.

Except in Calf No. 1140, the prescapular lymph node

of the side of inoculation enlarged, whereas, the prescapular lymph node of the side opposite to the site of inoculation did not.

Daily examination of blood smears failed to reveal the development of erythrocytic forms of the parasite. The development of macroschizonts is shown in Table 34. Daily biopsy of the prescapular lymph nodes on the side opposite to the site of inoculation were unsuccessful in revealing the presence of macroschizonts. The prepatent period and the period of patency of macroschizonts for individual calves and the mean in each group are presented in Table 33. In the second group Calf No. 1140 showed no macroschizonts. The means of the prepatent periods in the 2 groups were similar but the mean of the period of patency was considerably longer in the first group than in the second group.

The results of haematological studies on the vaccinated calves are given in Appendix Tables 8 and 10. The mean values for red cell counts, estimations of PCV and Hb. of the first group and those of the second group are presented in Figs. 13 and 14 respectively. Observations on red cell counts and estimation of PCV and Hb. revealed no significant changes in the second group. A slight decrease in the values of red cells, PCV and Hb. was observed in the calves of the first group. The changes in PCV and Hb. corresponded with variations of red cells. There were no significant changes observable in total and differential leucocyte counts in either group.

Challenge test.

The clinical symptoms in the vaccinated groups were limited to a slight rise of body temperature and a moderate enlargement of the local lymph nodes. The calves in the control group showed a severe reaction causing 2 of them to lie down for a few days.

The body temperatures from Day 10 to Day 30 after challenge of the vaccinated and the control groups are given in Appendix Table 6. The mean daily body temperatures of the vaccinated and the control groups are shown in Figs. 15 and 16, respectively. The thermal reactions are summarised in Table 29. Reference to Appendix Table 6 and Table 29 shows that the mean time to onset of fever in the vaccinated group was slightly longer than that in the control group. Duration of fever was considerably longer in the control group than in the vaccinated group viz. the mean of the duration of fever in the control group was 8.33 days, whereas the corresponding figure in the vaccinated group was 2.28 days. In the vaccinated group the body temperature did not rise, except in Calf No. 1140, to 104°F. The body temperature attained in the control group was higher than 104°F. In the control group the thermal reaction in Calf No. 1069 was considerably lower than in the other 2 calves. This contributed to a lowering of the mean values in the control group.

The local prescapular lymph nodes of the calves in the control group showed considerable enlargement. The

size of the prescapular lymph nodes in the vaccinated group also was slightly increased. This was most noticeable in Calf No. 1140.

Parasitological data are presented in Tables 30 and 31. The prepatent period before the appearance of erythrocytic forms and macroschizonts, the period of patency of macroschizonts and the maximum number of erythrocytic forms produced are given in Table 29. All the calves showed erythrocytic forms of the parasite. There was considerable difference between the mean of the number of erythrocytic forms per 100 red cells in the 2 groups. Erythrocytic forms in the vaccinated group did not rise above 0.2% whereas in the control group it rose to 6%. In the vaccinated group no schizonts could be detected in Calf No. 1116, but they were detected in the other animals. The period of patency of schizonts, which was very short in the vaccinated group, was very long in the control group.

The results of haematological studies are presented in Appendix Table 12. The mean values are given in Figs. 17, 18 and 19. In the vaccinated group there were no apparent changes in red cell counts, estimation of PCV and Hb. In the control group, results differed between Calf No. 1069 and the other 2 calves (Nos. 1121 and 1131). For clarity the mean of the values in Calves No. 1121 and No. 1131 and the values for Calf No. 1069 are presented separately. The loss of red cells in Calves No. 1121 and No. 1131 was intense and it led to anaemia with the

appearance of anisocytosis and poikylocytosis. The red cell counts commenced to rise in Week 5 after challenge, in these 2 calves. The changes in haemoglobin followed the trend of total red cells and PCV.

The data on the thermal and parasitic reactions were statistically analysed by the application of the t test. The results are given in Table 32. From this table it can be deduced that the thermal and parasitic reactions were significantly more severe in the control group than in the vaccinated group.

Experiment 6.2

Challenge test 12 months after vaccination.

Materials

Vaccination.

1 - Seventeen calves, 4 to 5 months old, as shown in Table 35, were divided into 2 groups. Group 1 consisted of 10 animals and Group 2 of 7 animals.

2 - The immunizing vaccine was the same as that described in the previous experiment. The method of vaccination and the dose were as described above. Only the first group was vaccinated.

Challenge.

1 - All the animals, the vaccinated and the controls, were challenged 12 months after vaccination.

2 - Strain D was used as the challenge strain. To prepare the inoculum for challenge, infected blood, which had been preserved at -70°C., was inoculated

into Calf No. 1167. At the peak of thermal and parasitic reactions one litre of blood was drawn from this calf into a beaker containing sodium citrate, as anticoagulant; Calf No. 1167 died 2 days after bleeding, of an acute theileriosis. Forty ml. of this infected blood were inoculated subcutaneously into the neck of each animal on the side shown in Table 35.

Observations

Vaccination.

1 - Body temperature.

2 - Blood smears.

3 - Palpation of the prescapular lymph nodes.

4 - Haematological studies.

5 - Lymph node biopsies.

All these observations were carried out as described for vaccination in Experiment 6.1.

Challenge test.

Complete observations as in the vaccination described above were made in 7 of the vaccinated animals and 7 controls. Calves Nos. 1122, 1124 and 1112 of the vaccinated group were temperature recorded only. Macroschizonts were counted as described in Experiment 5.1.

Results

Vaccination.

Daily body temperature of the calves, from Day 10 to Day 20 after vaccination, are recorded in Appendix Table 5.

The individual time to onset of fever and the means and the duration of, and maximum increase in, the body temperature of the calves are summarised in Table 33. There were considerable variations in the thermal responses of the animals.

Prescapular lymph nodes of the side of inoculation, in all the animals, enlarged. There was no corresponding enlargement on the side opposite to the site of inoculation.

Daily examination of blood smears revealed that erythrocytic forms of the parasite were undetectable. The times to the appearance of macroschizonts and their development are recorded in Table 34. Daily examination of the biopsy smears of the prescapular lymph nodes on the side opposite to the site of inoculation revealed no macroschizonts. The prepatent periods and the periods of patency of macroschizonts, in the local lymph nodes, for individual calves and the means are presented in Table 33. Reference to this table indicates that there were considerable variations in the parasitic reaction of the animals.

The results of haematological studies on the vaccinated calves are given in Appendix Tables 9 and 11. However, the mean values of the red cell counts, estimation of PCV and Hb. are charted in Fig. 20. Reference to Appendix Table 9 and Fig. 20 reveals no significant deviation from the normal ranges. No significant deviation from the normal ranges was recorded in total and differential counts of the leucocytes.

Challenge test.

All the control animals underwent severe symptoms of theileriosis, particularly Calf No. 1109. Two of the vaccinated animals manifested severe reactions to the challenge. In all the animals of the vaccinated group the parasitological reactions, the thermal reactions and the haematological changes were in order of severity. The parasitological reactions were the least and the haematological changes were the most severe.

The daily body temperatures of the vaccinated and the control animals, from Day 10 to Day 20 after challenge, are recorded in Appendix Table 7. The times of onset of fever, duration of pyrexia and the maximum increases in the body temperature of all the animals are summarised in Table 36. The mean daily body temperatures from Day 10 after challenge for the vaccinated and the control groups are charted in Figs. 21 and 22 respectively. The mean time to onset of fever in the vaccinated group was markedly longer than in the control group. The duration of pyrexia in the vaccinated group was significantly shorter than in the control group. The difference between the means of maximum increase in body temperature of the 2 groups was insignificant. Statistical analysis of this data by application of the t test is given in Table 37.

The parasitological data are presented in Tables 38 and 39. The mean times to appearance and the daily increase of schizonts and erythrocytic forms are plotted against mean daily body temperature of the vaccinated and

the control groups in Figs. 21 and 22 respectively. One of the animals, Calf No. 1087, in the vaccinated group did not show schizonts at all. The mean prepatent periods for schizonts and erythrocytic forms in the vaccinated group were markedly longer than in the control group. Reference to Table 36 indicates that the mean patent period for macroschizonts, in the animals that showed schizonts in the local lymph nodes, was significantly shorter in the vaccinated group than in the control group. The mean maximum percentages of erythrocytic forms in the control group were almost 3 times greater than in the vaccinated group (Table 36). In the vaccinated group Calf No. 1087, which did not show schizonts in the local lymph node, showed a maximum of 0.5% erythrocytic forms in the blood. Statistical analysis of the parasitological data is given in Table 37.

Haematological observations are recorded in Appendix Tables 13 and 14 for the vaccinated and the control groups respectively. The mean values of the red cell counts, PCV and Hb. for the vaccinated and the control groups are given in Figs. 23, 24 and 25 respectively. Reference to Appendix Table 13 indicates that 2 animals, Nos. 1091 and 1093, in the vaccinated group manifested very severe haematological changes and these contributed, considerably, to the lowering of the mean values in this group. The rest of the animals in the vaccinated group showed relatively mild changes. In the control group, referring to Appendix Table 14, one animal, No. 1109, which was older

than the rest, showed very severe changes. The rest of the animals underwent mild to severe reactions.

Table 28

Animals and inoculations in Experiment 6.1.

Animal No.	Group No.	Age in months at:		Side of inoculation at:	
		Vaccination	Challenge	Vaccination	Challenge
691	1	11.0	15.0	Right	Left
705	1	11.5	15.5	Right	Left
731	1	12.0	16.0	Right	Left
1108	2	5.5	9.5	Right	Left
1116	2	5.0	9.0	Right	Left
1134	2	4.5	8.5	Right	Left
1140	2	4.0	8.0	Right	Left
1069	3	-	8.0	-	Left
1121	3	-	8.0	-	Left
1131	3	-	8.0	-	Left

Animals in Groups 1 and 2 were vaccinated.

Animals in Group 3 were used in challenge as controls.

Table 29

Thermal and parasitic reactions of vaccinated and control calves challenged 4 months after vaccination.

Animal No.	Group	Prepatent period (days)		Patent Period Schizonts (days)	Maximum Erythrocytic forms%	Pyrexia		
		Schizonts	Erythrocytic forms			Day to onset	Duration (days)	Maximum (oF)
691	Vaccinated	20	20	1	0.2	19	3	103.2
705	"	20	21	1	0.2	19	2	103.2
731	"	19	22	1	0.1	19	1	103.1
1108	"	21	23	1	0.1	19	2	103.4
1116	"	-	19	-	0.2	20	1	103.2
1134	"	20	20	2	0.4	18	3	103.3
1140	"	18	21	2	0.2	18	4	104.6
Mean		19 ± 1.02	20 ± 1.34	1.14 ± 0.69	0.2 ± 0.10	$18^{+}-0.69$	2 ± 1.11	103.42 ± 0.41
1069	Control	17	18	2	6.0	16	5	104.6
1121	"	19	20	9	4.0	20	9	107.2
1131	"	18	19	10	8.0	18	11	106.3
Mean		18 ± 1	19 ± 1	7 ± 4.35	6 ± 2	18 ± 2	8 ± 3.05	106.03 ± 1.36

- = Schizonts were not detected.

Table 30

Erythrocytic forms per 100 RBC in vaccinated and control calves challenged 4 months after vaccination

Calf No.	Group	Days related to challenge																					
		-1	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1134	Vaccinated	-	-	-	-	-	-	-	-	-	-	-	0.1	0.2	0.2	0.3	0.4	0.4	0.4	0.4	0.4	0.4	0.4
1140	"	-	-	-	-	-	-	-	-	-	-	-	-	0.1	0.1	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
1116	"	-	-	-	-	-	-	-	-	-	-	0.1	0.1	0.1	0.2	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.1
1108	"	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
731	"	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
705	"	-	-	-	-	-	-	-	-	-	-	-	-	0.1	0.1	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
691	"	-	-	-	-	-	-	-	-	-	-	-	0.2	0.2	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1
1069	Control	-	-	-	-	-	-	-	-	-	0.5	1	4	4	5	5	6	6	6	6	6	5	5
1121	"	-	-	-	-	-	-	-	-	-	-	-	0.2	0.5	2	3	4	4	4	4	4	4	4
1131	"	-	-	-	-	-	-	-	-	-	-	1	5	6	7	8	8	8	8	8	8	6	6

- = Negative

Table 31

Percentages of macroschizonts in relation to lymphocytes in biopsy smears from the lymph nodes of vaccinated and control calves challenged 4 months after vaccination

Calf No.	Group	Days related to challenge																				
		10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1134	Vaccinated	-	-	-	-	-	-	-	-	-	-	10	0.1	-	-	....	....	....	....	....	....	....
1140	"	-	-	-	-	-	-	-	-	0.5	0.1	-	-	....	....	....	....	....	....	....	....	....
1116	"	-	-	-	-	-	-	-	-	-	-	-	-	-	-	....	....	....	....	....	....	....
1108	"	-	-	-	-	-	-	-	-	-	-	-	0.1	-	-	....	....	....	....	....	....	....
731	"	-	-	-	-	-	-	-	-	-	0.1	-	-	....	....	....	....	....	....	....	....	....
705	"	-	-	-	-	-	-	-	-	-	0	0.1	-	-	....	....	....	....	....	....	....	....
691	"	-	-	-	-	-	-	-	-	-	0	0.1	-	-	....	....	....	....	....	....	....	....
1069	Control	-	-	-	-	-	-	-	0.5	1.5	-	-	....	...	....	....	....	....	....	....	....	....
1121	"	-	-	-	-	-	-	-	-	-	1.5	5	11	23	40	50	24	10	10	-	-	-
1131	"	-	-	-	-	-	-	-	-	0.1	2	2	15	32.2	63	67	41	26	10	-	-	-

.... = Not carried out - = Negative

Table 32

Statistical analysis of thermal and parasitic reactions of the vaccinated and the control animals challenged 4 months after vaccination.

	Vaccinated group		Control group		D.F.	t	P
	Mean	S.D.	Mean	S.D.			
Time to onset of fever (days)	18	0.69	18	2	8	1.21	>0.05
Duration of fever (days)	2.28	1.11	8	3.05	8	5.48**	<0.001
Maximum body temperature °F.	103.42	0.41	106.03	1.36	8	5.78**	<0.001
Period before appearance of macroschizonts (days)	19.66	1.02	18	1	8	2.31*	<0.05
Days of patent period (macroschizonts)	1.14	0.69	7	4.35	8	5.28**	<0.001
Period before appearance of erythrocytic forms (days)	20.85	1.34	19	1	8	2.13	>0.05
Maximum % of erythrocytic forms	0.20	0.10	6	2	8	14.50**	<0.001

* significantly different. ** highly significantly different.

Table 33

Thermal and parasitic reactions of calves vaccinated with frozen tissue culture vaccine in Experiments 1 and 2.

		Animal No.	Pyrexia			Macroschizonts	
			Day to onset	Duration (days)	Maximum (°F.)	Prepatent period (days)	Patent period (days)
Experiment 1	Group 1	691	15	3	105.2	13	6
		705	13	5	105.2	14	5
		731	11	4	105.3	10	8
	Mean		13±2	4±1	105.23±0.06	12.33±2.08	6.33±1.52
	Group 2	1108	10	3	103.8	12	3
		1116	10	8	105.4	13	1
		1134	17	1	103.6	12	4
		1140	16	2	103.6	-	-
	Mean		13.25±3.75	3.50±3.10	104.10±0.87	12.33±0.57	2.33±1.52
Experiment 2		1087	13	5	104.8	14	4
		1091	14	5	104.8	16	3
		1093	13	6	105.0	12	8
		1099	13	5	104.8	11	6
		1112	13	2	103.3	14	1
		1122	14	5	104.2	12	3
		1124	11	2	103.2	12	1
		1128	15	6	105.4	16	4
		1130	17	1	103.0	14	1
		1146	13	2	104.2	12	4
	Mean		13.60±1.57	3.90±1.91	104.27±0.84	13.30±1.71	3.50±2.27

- = Schizonts could not be detected.

Table 34

Percent macroschizonts in relation to lymphocytes in biopsy smears from vaccinated calves in Experiment 1 and 2.

		Animal No.	Days related to vaccination										
			10	11	12	13	14	15	16	17	18	19	20
Experiment 1	Group 1	691	-	-	-	1.0	3.0	3.0	2.0	5.0	0.5	-	-
		705	-	-	-	-	1.0	4.0	8.0	3.0	0.5	-	-
		731	0.1	0.1	4.0	10.0	3.0	1.5	0.5	0.2	-	-	..
	Group 2	1108	-	-	0.2	2.5	4.3	-	-	..	..	..	..
		1116	-	-	-	1.0	-	-	..	..	..	..	..
		1134	-	-	0.3	16.0	13.0	0.1	-	-	..	..	..
		1140	-	-	-	-	-	-	-	-	-	-	-
Experiment 2		1087	-	-	-	2.7	13.5	37.6	3.5	-	-	..	..
		1091	-	-	-	-	-	-	0.2	2.0	0.2	-	-
		1093	-	-	0.5	1.5	6.5	30.0	94.5	53.0	10.0	1.0	-
		1099	-	1.0	2.5	7.0	19.5	5.0	1.0	-	-	..	..
		1112	-	-	-	-	<0.1	-	-	..	..	..	..
		1122	-	-	-	<0.1	<0.1	<0.1	-	-	..	..	..
		1124	-	-	<0.1	-	-	..	..	..	..	..	..
		1128	-	-	-	-	-	-	0.1	1.5	15.5	0.2	-
		1130	-	-	-	-	0.1	-	-	..	..	..	..
		1146	-	-	2.0	15.8	62.3	5.1	-	-	..	..	..

- = No schizonts could be detected. .. = Not carried out

Table 35

Animals and inoculations in Experiment 6.2.

Animal No.	Group No.	Age in months at:		Side of inoculation at:	
		Vaccination	Challenge	Vaccination	Challenge
1087	1	5.5	17.5	Right	Left
1091	1	5.0	17.0	Right	Left
1093	1	5.0	17.0	Right	Left
1099	1	5.0	17.0	Right	Left
1112	1	5.0	17.0	Right	Right
1122	1	5.0	17.0	Right	Right
1124	1	5.0	17.0	Right	Right
1128	1	5.0	17.0	Right	Right
1130	1	4.5	16.5	Right	Right
1146	1	4.5	16.5	Right	Right
1109	2	-	17.0	-	Right
1241	2	-	4.0	-	Right
1243	2	-	4.0	-	Right
1245	2	-	4.0	-	Right
1247	2	-	4.0	-	Right
1249	2	-	4.0	-	Right
1251	2	-	4.0	-	Right

Animals in Group 1 were vaccinated.

Animals in Group 2 were used in challenge as controls.

Table 36

Thermal and parasitic reactions of vaccinated and control animals challenged 12 months after vaccination.

Animal No.	Group	Prepatent period (days)		Patent period schizonts (days)	Maximum % erythrocytic forms	Pyrexia		
		Schizonts	Erythrocytic forms			Day to onset	Duration (days)	Maximum (oF.)
1087	Vaccinated	-	19	-	0.5	17	3	103.6
1091	"	19	20	3	15.0	17	9	106.4
1093	"	19	17	3	8.0	19	7	106.2
1099	"	21	17	1	4.0	18	5	105.6
1112*	"					17	6	104.2
1122	"	18	17	3	5.0	17	4	105.0
1124*	"					16	4	105.6
1128	"	17	17	3	5.0	17	6	106.2
1130*	"					17	4	105.2
1146	"	18	19	2	1.0	18	5	104.2
Mean		18.66±1.36	18±1.29	2.5±0.83	5.5±4.90	17.3±0.82	5.3±1.76	105.22±0.96
1109	Control	12	14	10	30.0	12	13	107.0
1241	"	16	16	6	8.0	14	8	105.6
1243	"	15	16	4	7.0	14	9	104.6
1245	"	16	16	6	15.0	14	9	105.0
1247	"	16	16	5	15.0	15	8	106.7
1249	"	15	14	10	25.0	13	13	107.8
1251	"	14	15	8	12.0	13	9	106.8
Mean		14.85±1.46	15.28±0.94	7±1.46	16±8.56	13.57±0.98	9.85±2.19	106.21±1.19

* Only temperatures were recorded.

Table 37

Statistical analysis of thermal and parasitic reactions of the vaccinated and the control animals challenged 12 months after vaccination.

	Vaccinated group		Control group		D.F.	t	P
	Mean	S.D.	Mean	S.D.			
Time to onset of fever (days)	17.30	0.82	13.57	0.98	15	8.00**	<0.001
Duration of fever (days)	5.30	1.76	9.85	2.19	15	4.77**	<0.001
Maximum body temperature (°F.)	105.22	0.96	106.21	1.19	15	1.90	>0.05
Period before appearance of macroschizonts (days)	18.66	1.36	14.85	1.46	11	4.82**	<0.001
Days of Patent period (macroschizonts)	2.00	0.83	7.00	2.38	11	4.81**	<0.001
Period before appearance of erythrocytic forms (days)	18.00	1.29	15.28	0.94	12	4.53**	<0.001
Maximum % of erythrocytic forms	5.50	4.90	16.00	8.56	12	2.91*	<0.05 >0.01

* Significantly different ** highly significantly different

Table 38

Percent macroschizonts in relation to lymphocytes in biopsy smears from vaccinated and control animals challenged 12 months after vaccination.

Animal No.	Group	Days related to challenge																				
		10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1087	Vaccinated	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1091	"	-	-	-	-	-	-	-	-	-	0.2	10.0	5.0	-	-	..	..	..	..	..	..	..
1093	"	-	-	-	-	-	-	-	-	-	0.1	1.0	1.0	-	-	..	..	..	..	..	..	..
1099	"	-	-	-	-	-	-	-	-	-	-	0.1	1.0	-	-	..	..	..	..	..	..	..
1022	"	-	-	-	-	-	-	-	-	0.9	0.5	0.1	-	-	-	..	..	..	..	..	..	..
1028	"	-	-	-	-	-	-	-	0.5	1.0	1.0	-	-	..	..	..	..	..	..	..	..	..
1046	"	-	-	-	-	-	-	-	-	1.0	0.5	-	-	..	..	..	..	..	..	..	..	..
1109	Control	-	-	1.0	5.0	10.0	25.0	30.0	80.0	95.0	85.0	50.0	0.1	-	-	..	..	..	..	..	..	..
1241	"	-	-	-	-	-	-	0.1	0.1	2.0	2.0	1.0	0.1	-	-	..	..	..	..	..	..	..
1243	"	-	-	-	-	-	0.2	0.4	0.5	1.0	-	-	..	..	..	..	..	..	..	..	..	..
1245	"	-	-	-	-	-	-	5.0	10.0	15.0	10.0	5.0	2.0	-	-	..	..	..	..	..	..	..
1247	"	-	-	-	-	-	-	0.1	10.0	50.0	5.0	1.0	-	-	..	..	..	..	..	..	..	..
1249	"	-	-	-	-	-	1.0	16.0	16.0	30.0	15.0	5.0	2.0	0.1	0.1	0.1	-	-	..	..	..	..
1251	"	-	-	-	-	2.0	5.0	10.0	10.0	35.0	0.1	0.1	0.1	-	-	..	..	..	..	..	..	..

- = Negative .. = Not carried out

Table 39

Erythrocytic forms per 100 RBC in vaccinated and control animals challenged 12 months after vaccination.

Animal No.	Group	Days related to challenge										
		-1	10	11	12	13	14	15	16	17	18	19
1087	Vaccinated	-	-	-	-	-	-	-	-	-	-	0.1
1091	"	-	-	-	-	-	-	-	-	-	-	-
1093	"	-	-	-	-	-	-	-	-	0.1	0.1	0.2
1099	"	-	-	-	-	-	-	-	-	0.1	0.1	1.0
1122	"	-	-	-	-	-	-	-	-	0	0.1	2.0
1128	"	-	-	-	-	-	-	-	-	0.1	1.0	2.0
1146	"	-	-	-	-	-	-	-	-	-	-	0.1
1109	Control	-	-	-	-	-	5	10.0	15.0	16.0	20.0	25.0
1241	"	-	-	-	-	-	-	-	2.0	2.0	2.0	2.0
1243	"	-	-	-	-	-	-	-	0.1	0.1	1.0	2.0
1245	"	-	-	-	-	-	-	-	0.1	1.0	2.0	2.0
1247	"	-	-	-	-	-	-	-	4.0	8.0	9.0	10.0
1249	"	-	-	-	-	-	0.1	2.0	5.0	5.0	6.0	8.0
1251	"	-	-	-	-	-	-	2.0	4.0	5.0	6.0	8.0

- = Negative

Table 39 (cont'd)

Erythrocytic forms per 100 RBC in vaccinated and control animals challenged 12 months after vaccination.

Animal No.	Group	Days related to challenge										
		20	21	22	23	24	25	26	27	28	29	30
1087	Vaccinated	0.1	0.1	0.2	0.2	0.5	0.5	0.5	0.5	0.5	0.5	0.5
1091	"	0.5	1.0	5.0	10.0	15.0	10.0	10.0	5.0	5.0	5.0	6.0
1093	"	1.0	5.0	6.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0
1099	"	2.0	2.0	2.0	2.0	3.0	4.0	4.0	2.0	2.0	2.0	2.0
1122	"	4.0	5.0	5.0	5.0	4.0	2.0	1.0	1.0	1.0	1.0	0.5
1128	"	5.0	5.0	5.0	5.0	5.0	4.0	4.0	3.0	2.0	2.0	1.0
1146	"	0.2	1.0	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
1109	Control	30.0	30.0	30.0	30.0	30.0	25.0	25.0	25.0	15.0	10.0	10.0
1241	"	2.0	2.0	4.0	5.0	5.0	6.0	7.0	8.0	8.0	5.0	2.0
1243	"	2.0	4.0	4.0	5.0	5.0	5.0	5.0	5.0	7.0	7.0	5.0
1245	"	5.0	8.0	12.0	15.0	20.0	15.0	10.0	8.0	4.0	4.0	4.0
1247	"	12.0	14.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	10.0	10.0
1249	"	9.0	10.0	15.0	18.0	20.0	25.0	20.0	15.0	10.0	8.0	6.0
1251	"	10.0	12.0	10.0	12.0	8.0	10.0	10.0	4.0	6.0	7.0	7.0

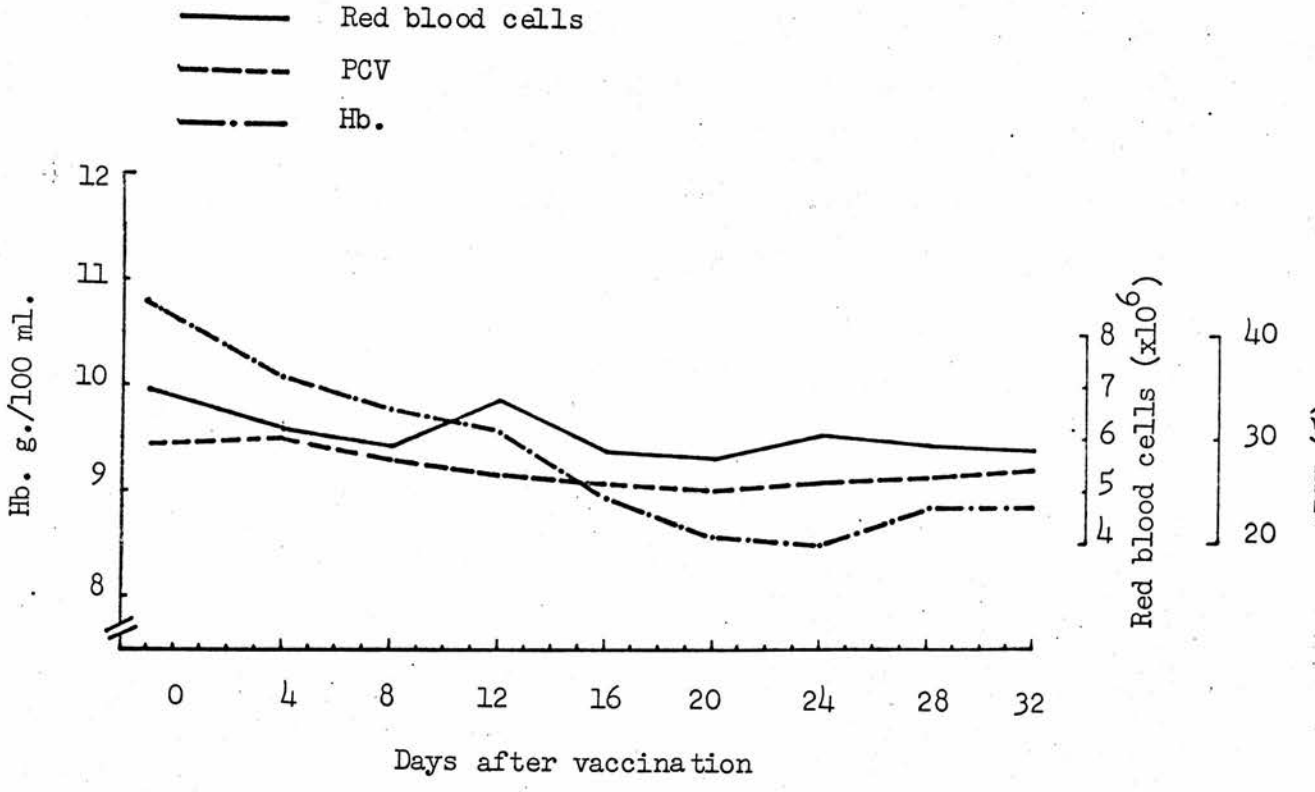

Fig. 13 Mean red blood cell counts, estimations of PCV and Hb. of calves in first group Experiment 6.1.

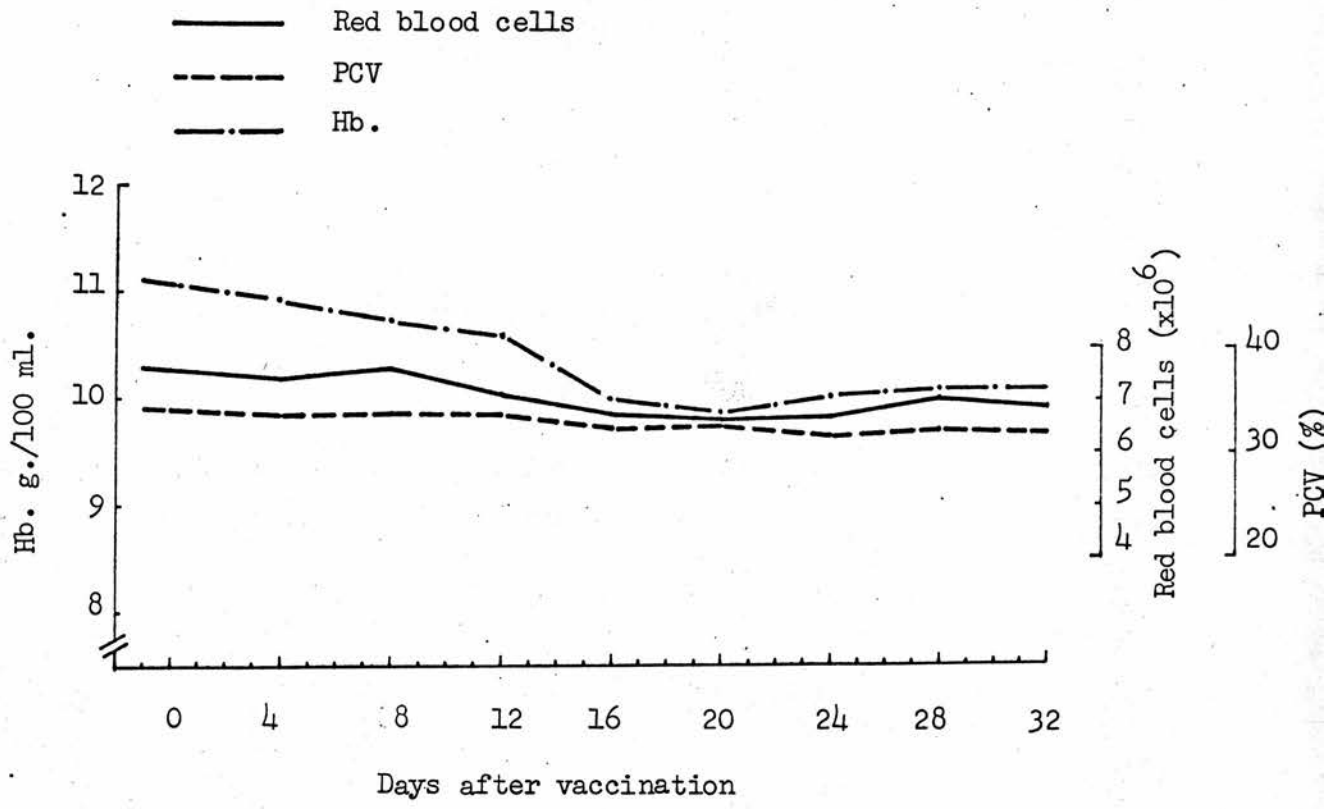

Fig. 14 Mean red blood cell counts, estimations of PCV and Hb. of calves in second group Experiment 6.1.

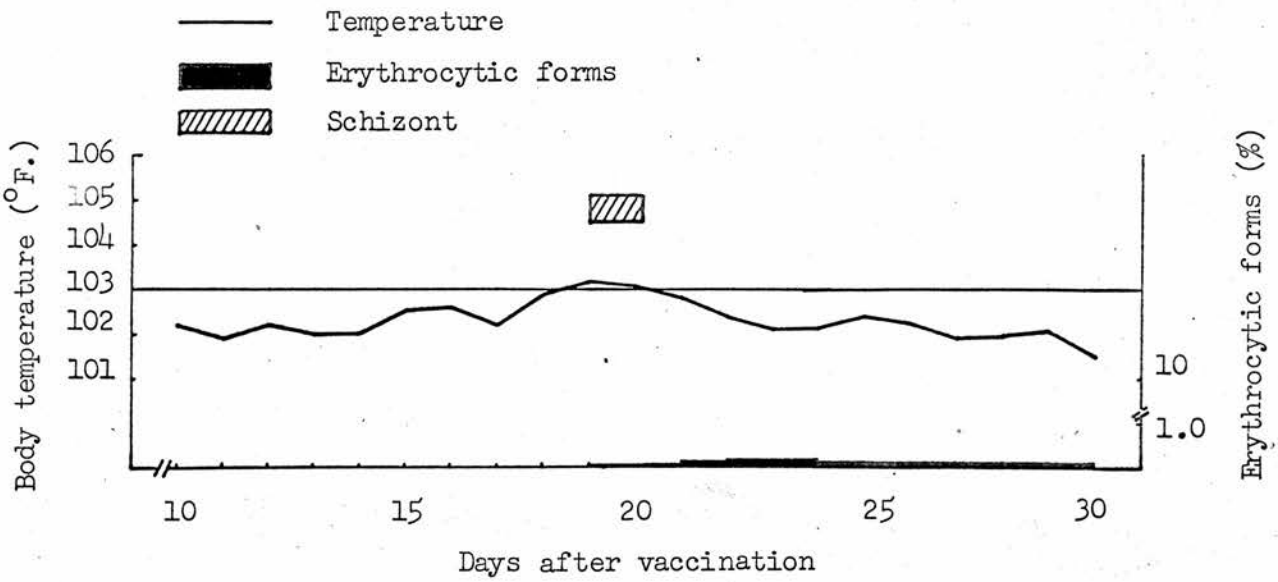

Fig. 15 Mean daily body temperatures, erythrocytic forms and schizonts in vaccinated calves challenged 4 months after vaccination.

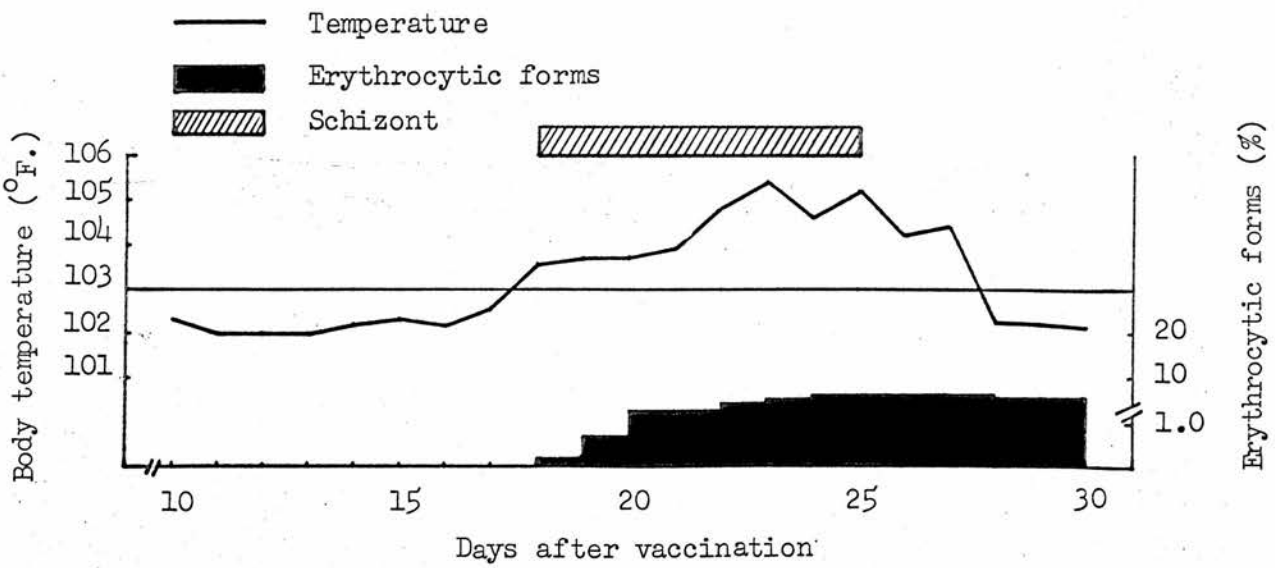

Fig. 16 Mean daily body temperatures, erythrocytic forms and schizonts in control calves challenged 4 months after vaccination.

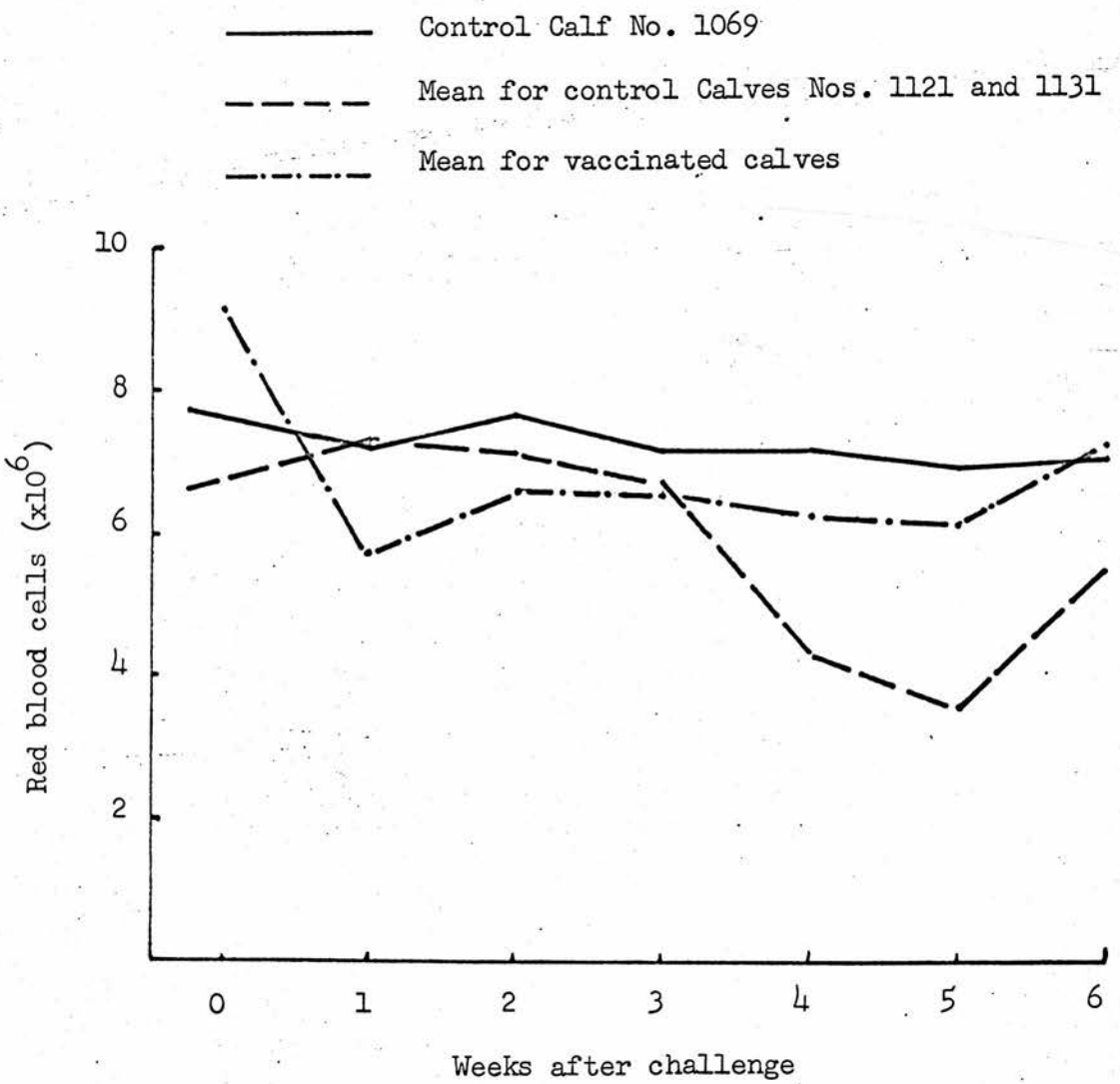

Fig. 17 Mean red blood cell counts of vaccinated and control calves challenged 4 months after vaccination.

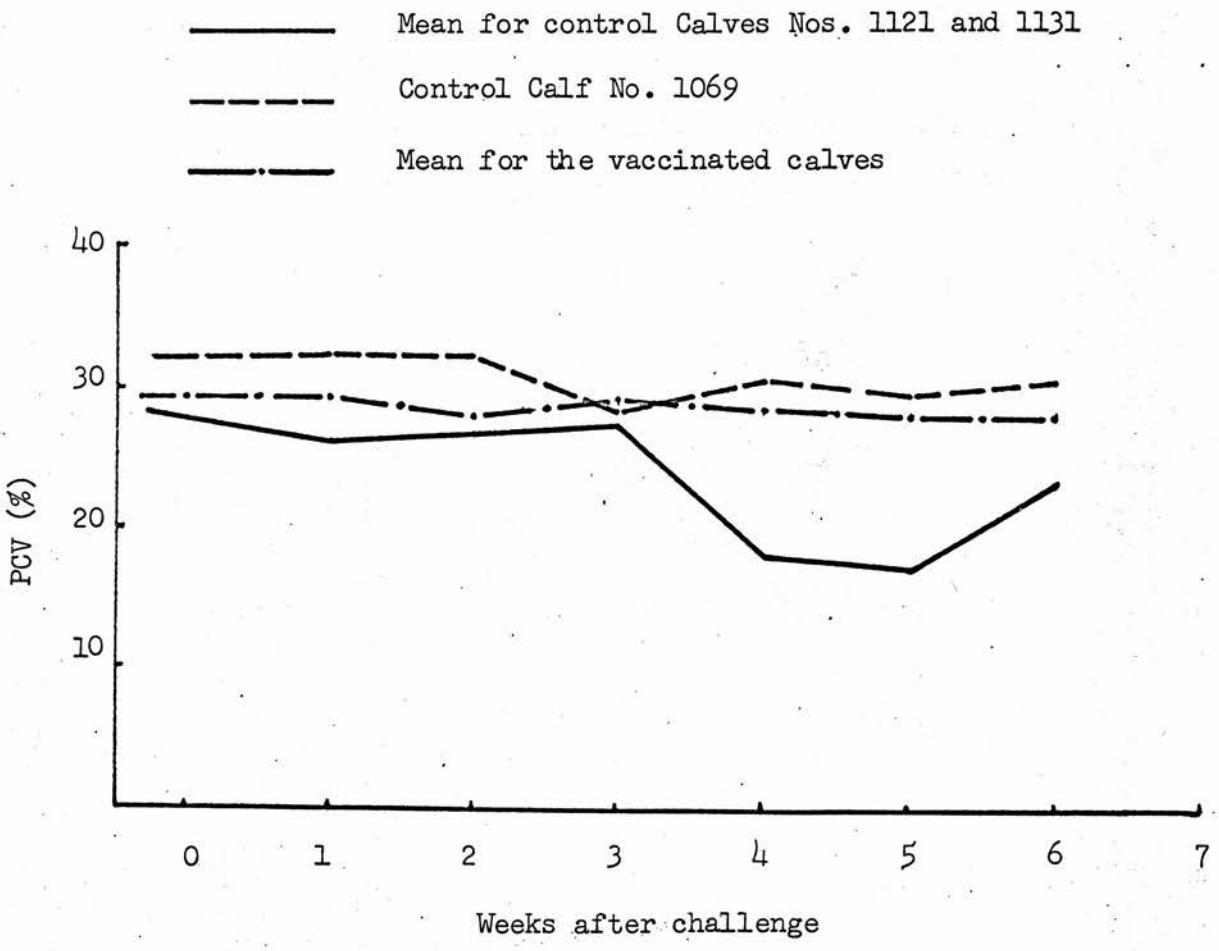

Fig. 18 Mean of estimations of PCV of vaccinated and control calves challenged 4 months after vaccination.

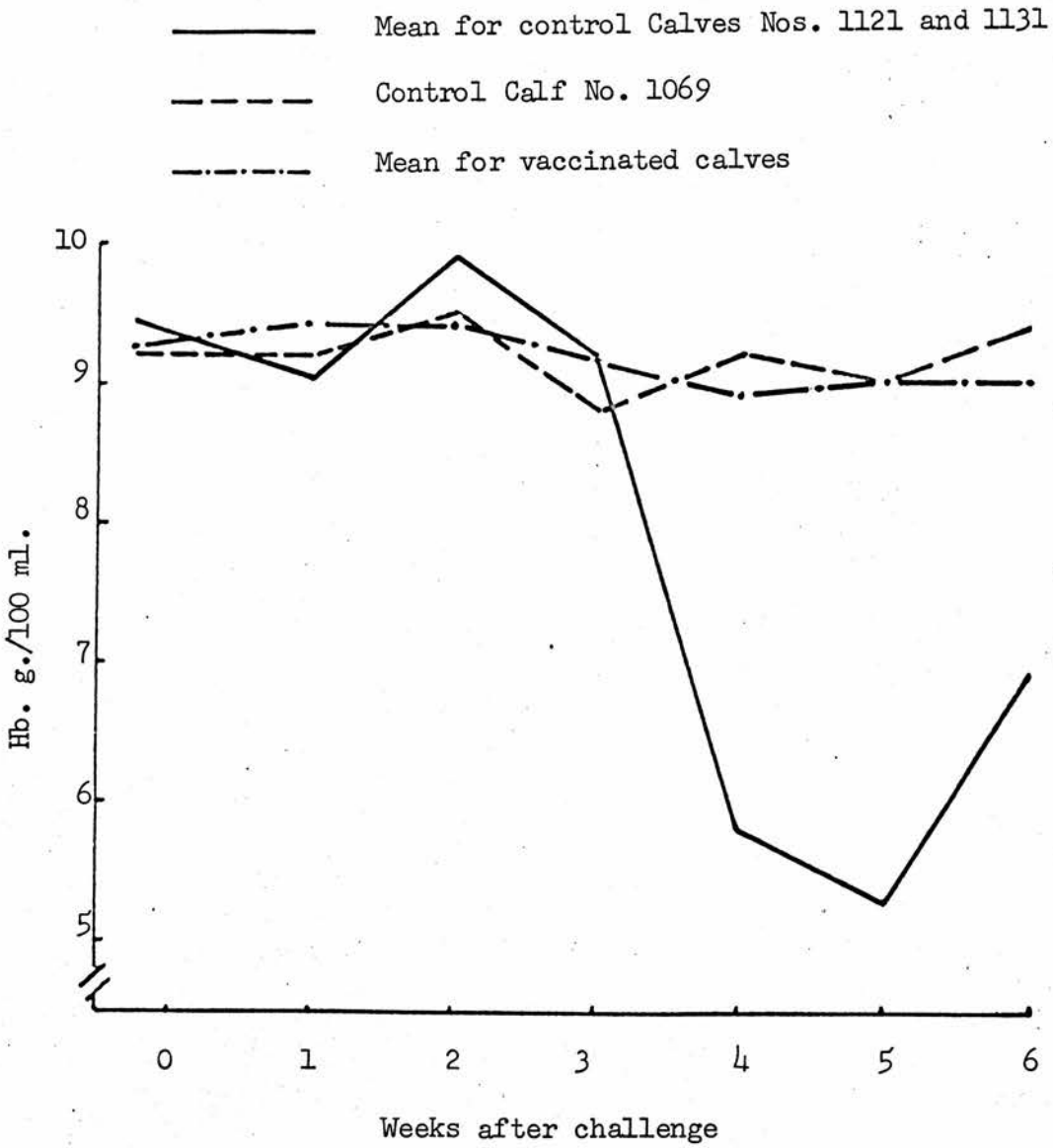

Fig. 19 Mean of estimations of Hb. of vaccinated and control calves challenged 4 months after vaccination.

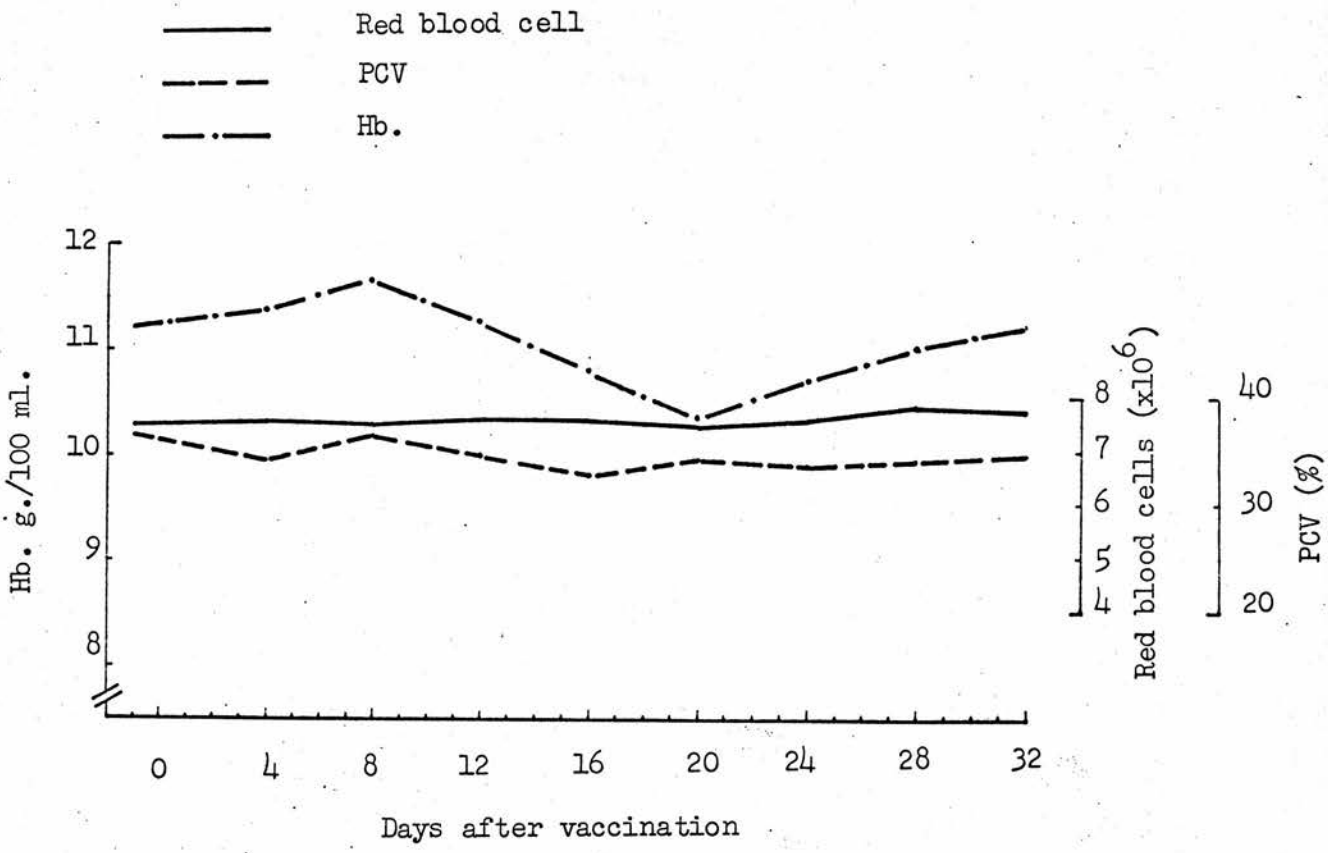

Fig. 20 Mean of red blood cell counts, estimations of PCV and Hb. of calves vaccinated in Experiment 6.2.

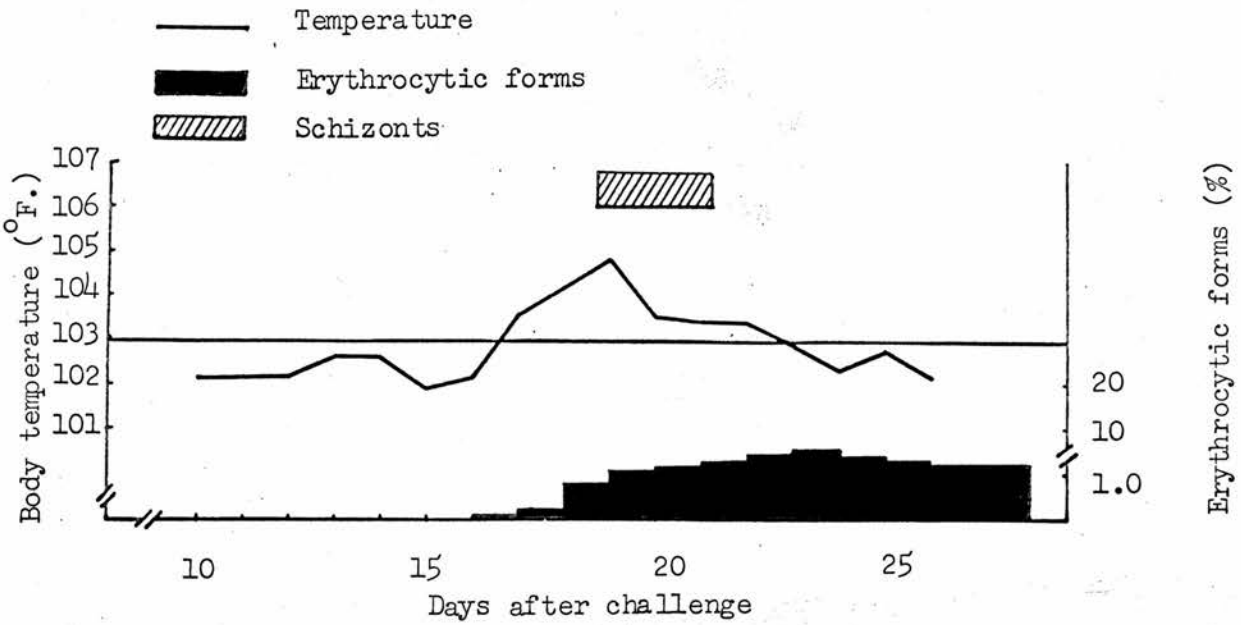

Fig. 21 Mean daily body temperatures, erythrocytic forms and schizonts in vaccinated calves challenged 12 months after vaccination.

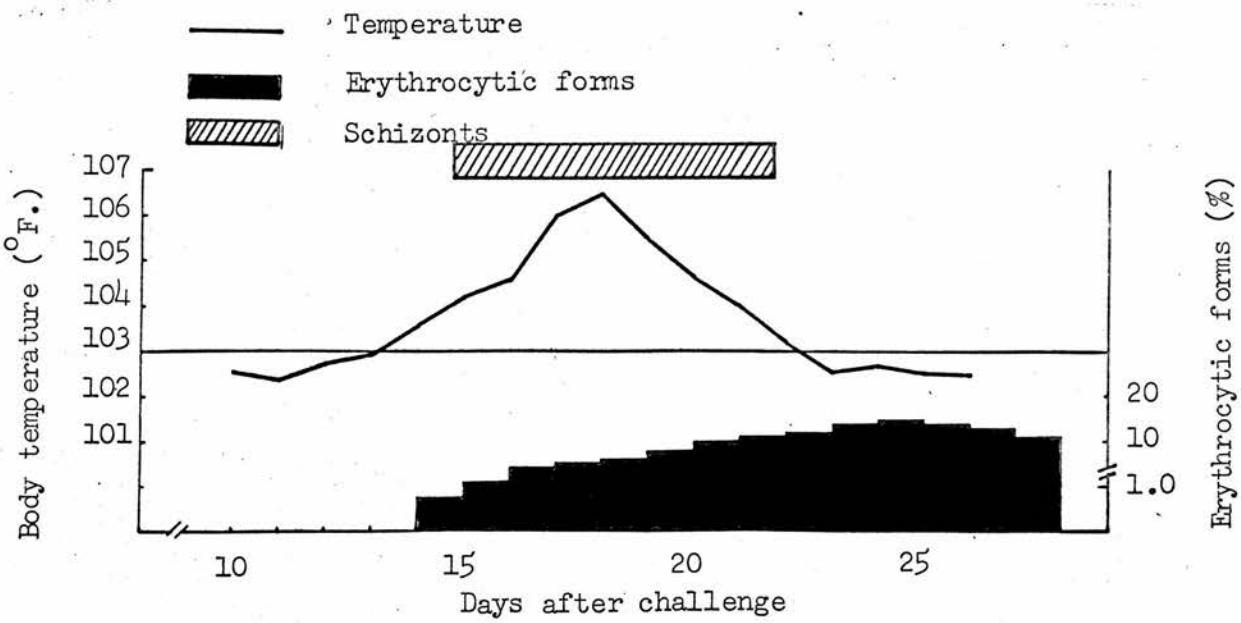

Fig. 22 Mean daily body temperatures, erythrocytic forms and schizonts in control calves challenged 12 months after vaccination.

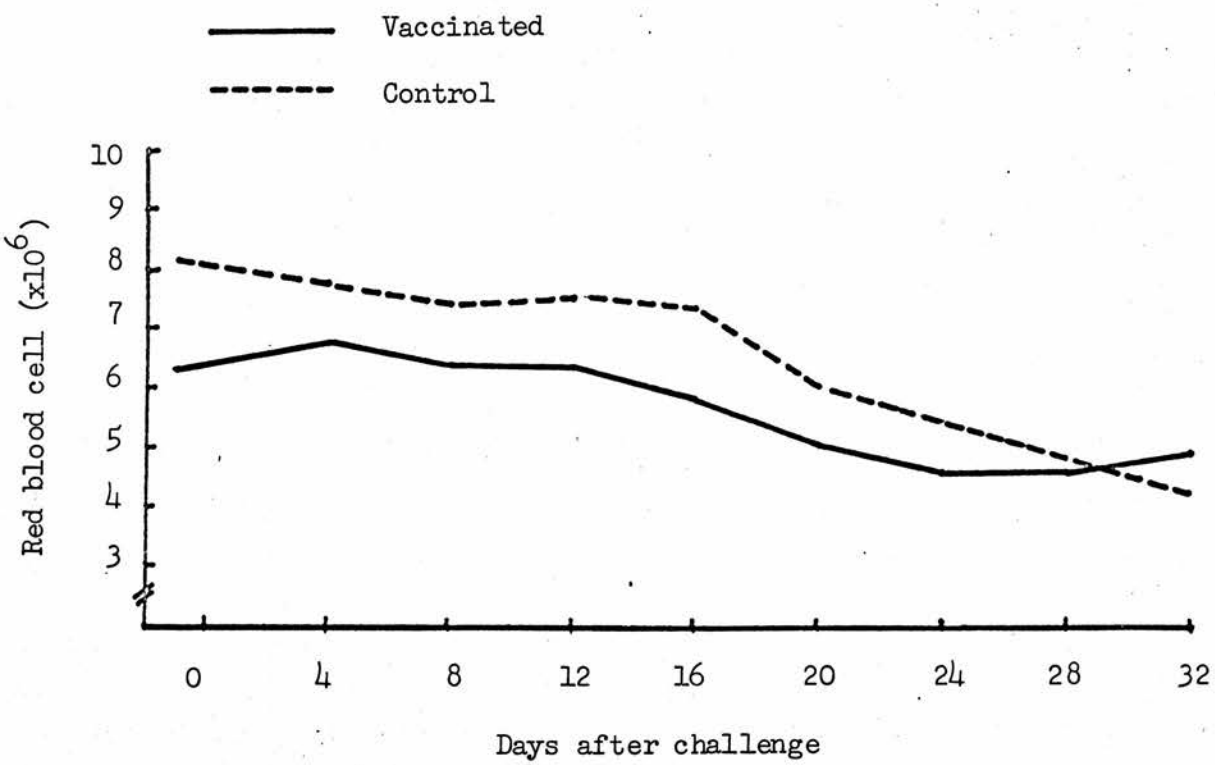

Fig. 23 Mean % red blood cell counts of vaccinated and control cattle challenged 12 months after vaccination.

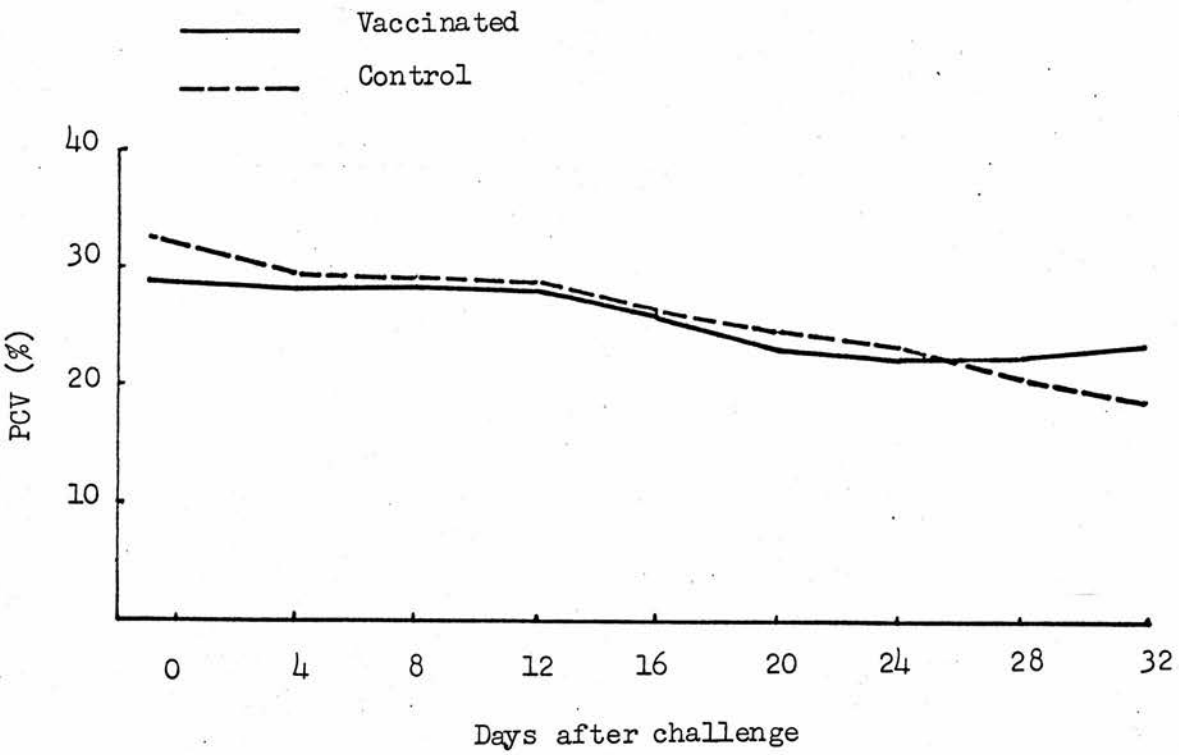

Fig. 24 Mean of estimations of PCV of vaccinated and control cattle challenged 12 months after vaccination.

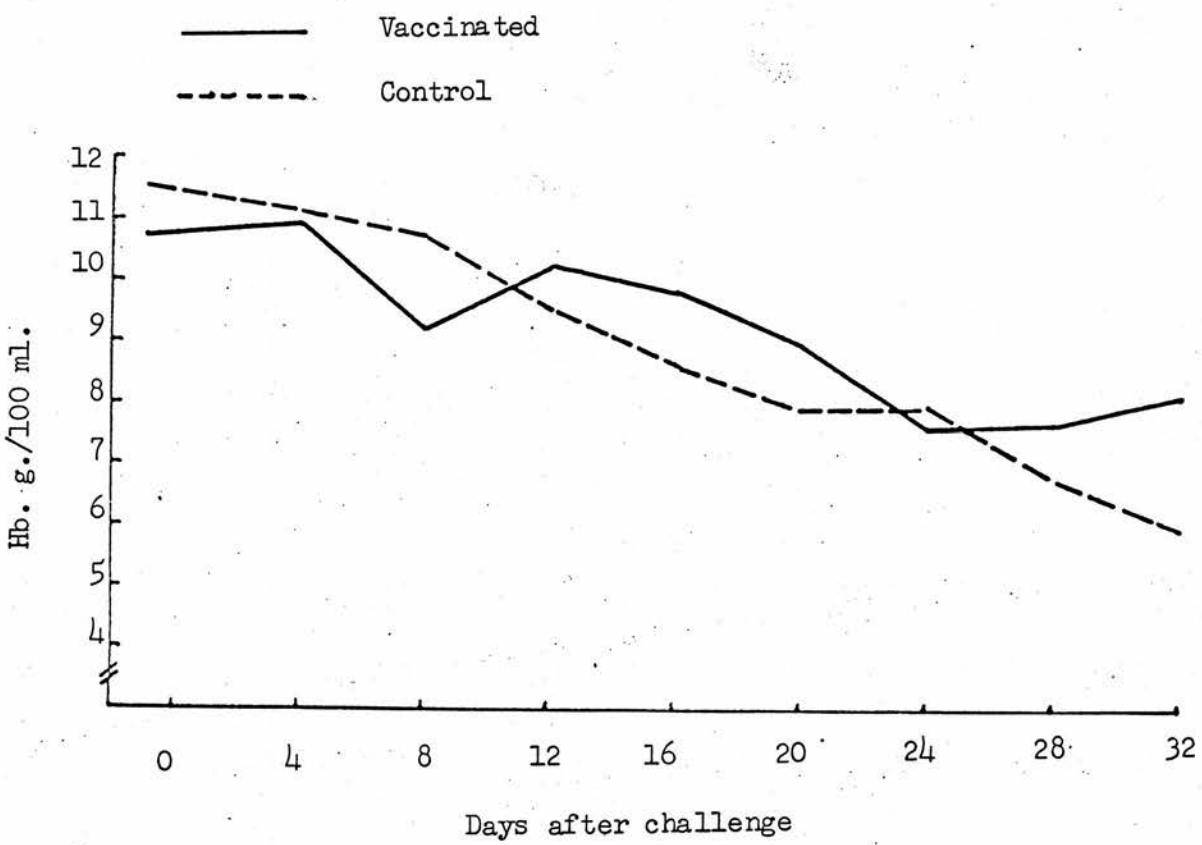

Fig. 25 Mean of estimations of Hb. of vaccinated and control cattle challenged 12 months after vaccination.

DISCUSSION AND CONCLUSIONS

Haematological studies of the vaccinated calves, except in the older animals of the first group of Experiment 6.1, indicated that no measurable anaemia developed following the mild reaction in vaccinated animals. The mean preinoculation value of PCV in animals of the first group of Experiment 6.1 was low, but this was considered to be the normal value at this age (Schalm 1965). Although there were losses of up to 3 g. Hb. per 100 ml. of blood in these animals, the values were within the normal range (Schalm 1965). However, this fall could not definitely be attributed to the response to vaccination as it appeared many days prior to the commencement of the reactions.

Strain C, which killed Calf No. 1143 the donor of the first challenge inoculum, was not used in the second challenge as it caused no mortality in the control group. In the second challenge test Strain D which had been isolated from a fatal case of theileriosis was used, but this too caused no mortality in the control group, although some of the reactions were very severe.

The immunity, in the vaccinated animals, in the first challenge test was significantly high and prevented the appearance of clinical symptoms. The immune response in the second challenge test was measured by its marked effect on the means of the length of time to onset of fever, the duration of pyrexia, the length of prepatent period for erythrocytic forms and macroschizonts, the patent period

for macroschizonts and the maximum number of erythrocytic forms per 100 red cells produced. In the control group the reaction of animal No. 1109 was more severe than the rest of the animals which were younger.

Apparently the degree of parasitic and thermal reaction in response to vaccination had little or no bearing on the degree of immunity to challenge. This was clearly observed in the second challenge test. Calf No. 1093 which had shown the most severe reaction to the vaccine, with 8 days patency of macroschizonts, showed also a very severe reaction to the challenge. In the same challenge test Calf No. 1112 which had shown the mildest reaction to vaccination showed a very mild thermal reaction to challenge.

The non-appearance of macroschizonts in Calf No. 1116, at the first challenge, and Calf No. 1087, at the second challenge, did not mean that schizonts failed to develop in these animals as the erythrocytic forms which originate from schizonts, could be detected in both animals. It can only be said that the immune status of the animals prevented schizonts from multiplying freely and they were destroyed before they reached such numbers as to become detectable in biopsy smears. Irrespective of the degree of immunity in each calf, in the first and second challenges, the immune response became effective only sometime after the development of schizonts of the challenge strain.

In the first challenge, haematological changes in the vaccinated group were of a very low order, whereas in the

control group 2 animals showed severe haematological changes. Calf No. 1069, in the control group, which did not show severe thermal or schizontal reactions showed 6% of infected red cells. Inspite of this, which was of greater degree than in Calf No. 1121 of the same group, the haematological changes were considered to be slight. In the second challenge test the haematological changes, in the control group, differed markedly between animal No. 1109 in which 30% of red cells became infected and the rest which were younger, *viz*. PCV in this animal dropped to 12% at the end of the experiment, whereas Calf No. 1249 which showed 25% infection of red cells was, haematologically, much less affected. Moreover, in the vaccinated group animals Nos. 1093 and 1091 which showed maxima of 8% and 15% infected red cells respectively, suffered an anaemia as intense as that of animal No. 1109, in the control group. Sergent *et al*. (1945) stated "The red cells do not seem to be destroyed by the presence of the erythrocytic forms inside them, apparently they are carried as inert bodies, but still theileriosis is accompanied by anaemia no doubt due to damage caused in the haemopoietic system". Adler and Ellenbogen (1934) observed that in the recovered calves the blood picture immediately after the height of the infection was quite different from that of recovered adults at a similar stage of the disease. In the latter there was usually an aplastic anaemia, which in some cases lasted for a few months and the convalescence was very slow. Hill and Matson (1970) studied the haematology of

experimental T.lawrencei infection and expressed the opinion that it was difficult to assess the influence of these forms of the parasite on the dramatic blood changes that took place. It is speculated that the degree of anaemia is not closely related to the percentage of the infected red cells and apparently is associated with a host defence mechanism which is more rigorous in older animals.

Chapter 7

DISCUSSION

The present studies were designed to determine the duration of immunity engendered in bovine animals following vaccination with T.annulata tissue culture vaccine.

Subjects such as the cultivation of infected lymphoid cells, the isolation and attenuation of strains of T.annulata and the storage of infected lymphoid cells, which would lead to the preparation of a standard vaccine against tropical theileriosis had to be investigated further as the available information was inadequate. After defining the method of preparing the vaccine a series of experiments was carried out in order to standardize the methods of preservation and administration of such a vaccine.

The experiments were designed to compare the effects of vaccination with fresh and frozen vaccine, to determine the optimum dose and route of administration to be used and to investigate the variations in response to vaccination due to the age of the subject animal. The determination of the duration of immunity following vaccination was investigated by carrying out challenge tests, 4 and 12 months after vaccination. The results of this study, therefore, will be discussed in that order.

1 - Cultivation of infected lymphoid cells.

Mixed cultures of monolayers of fibroblasts and

lymphoid cells infected with T.annulata schizonts were successfully achieved by Tsur and Adler (1962). These authors used Earle's solution containing bovine serum and yeast. Hulliger (1965) cultivated T.annulata infected lymphoid cells in association with BHK cells, using Eagle's medium supplemented with 10% calf serum and 10% of a solution of tryptose phosphate broth (2% stock solution). This author did not explain the reason for the addition of tryptose phosphate broth to the Eagle's medium but expressed the opinion that infected lymphoid cells could not be grown in the absence of fibroblasts. Malmquist, Nyindo and Brown (1970) for cultivation of T.parva in tissue culture used Eagle's minimum essential medium (MEM) with Earle's salt base supplemented with 20% foetal calf serum and 0.100 g. L - - asparagine per litre. They did not state whether the addition of L - - asparagine to the medium was essential or had any contributory effect in increasing the rate of growth of T.parva infected lymphoid cells. The author of this thesis (unpublished work) repeatedly and unsuccessfully attempted to grow infected lymphoid cells in mixed as well as suspension cultures using Hanks' or Earle's salt solutions supplemented with various percentages of bovine serum, lactalbumin hydrolysate and yeast extract (LY). Hooshmand-Rad and Hashemi-Fesharki (1968) using Eagle's medium isolated T.annulata infected lymphoid cells in mixed culture and subsequently grew them in suspension culture. For this reason, the medium chosen for use in the present study was

Eagle's based on Hanks' salt solution. However, in the course of this work it was found that when LY were added to Eagle's medium the growth of infected lymphoid cells improved considerably. The amount of LY for optimum growth of infected lymphoid cells was determined as 40 ml. of a solution containing 25 g. lactalbumin and 5 g. yeast extract and this was the amount included in the definitive medium designated modified Eagle's medium and in vaccine production. When cultures with 10^5 cells/ml. were set up, using this medium, the cells increased in numbers almost 9 fold after 96 hours incubation at 37°C. This was more than twice the number that could be obtained when Eagle's medium without LY was used. Moulton, Krauss and Malmquist (1971a and 1971b) who studied T.parva infected lymphoid cells in suspension culture used 20% foetal calf serum in the medium for the growth of these cells and considered the quality and quantity of the serum were critical in their work. Hooshmand-Rad and Hashemi-Fesharki (1968) used 10% calf serum in the medium for suspension culture of T.annulata infected lymphoid cells. In the present work it was found that increasing the percentage of calf serum content of the modified Eagle's medium from 10% to 20% had no apparent favourable effect on the cultivation of the cells. Moreover, when calf serum in modified Eagle's medium was replaced by sheep or horse serum no adverse effect on propagation of infected lymphoid cells was observed. This confirmed the work conducted by Brocklesby and Hawking (1958) who observed that the growth

of T.annulata in explant cultures was not affected adversely when they replaced bovine serum with equine serum. Infected lymphoid cells grew readily, using modified Eagle's medium, even when very low numbers of cells were originally implanted. This was found to be useful in the isolation of strains of T.annulata in tissue culture. In cultures set up from biopsy materials obtained from calves, at the time of thermal and parasitic reactions to T.annulata infection, infected lymphoid cells established themselves in 7 days, whereas, at this stage of culture, fibroblasts had only just commenced to grow. Thus, the independent growth of infected lymphoid cells at the time of isolation was not as described by previous investigators. For example, Hulliger (1965) considered that the presence of fibroblasts was essential for isolation and growth of Theileria infected lymphoid cells. Hooshmand-Rad and Hashemi-Fesharki (1968) stated that for the isolation of T.annulata infected lymphoid cells and prior to initiation of suspension culture the presence of fibroblasts was necessary. Moulton et al. (1971b) stated that for suspension culture of T.parva infected lymphoid cells 4 to 5 weeks had to elapse before they could be transformed from reticulum cells into lymphoblast cells and become established in suspension cultures. In the present work the rapidity with which infected lymphoid cells established themselves led the author of this thesis to the conclusion that the isolation of these cells in tissue culture was merely a transplantation and not transformation.

2 - Attenuation

Sergent et al. (1927b and 1927c) attempted, unsuccessfully, to attenuate strains of T.annulata using different methods, such as passage, in susceptible calves, maintenance of infected ticks at high temperatures and the addition of various chemicals to the suspensions of organs harbouring schizonts. Pipano (1965) also confirmed that serial passages in susceptible calves did not attenuate the strains of T.annulata. Tsur and Pipano (1966) reported the attenuation of T.annulata in tissue culture. Tissue culture for attenuation of protozoa has been used by other investigators. Weiss and De Giusti (1966) were able to cultivate Plasmodium berghei in liver explants of infected rats in the presence of hamster serum. After a number of subpassages the parasite was rendered harmless to white mice but inoculated animals were immune against virulent strains. Pipano and Tsur (1966) described the mode of attenuation of 2 strains of T.annulata. They showed that one of the 2 strains became completely attenuated after 8 months in tissue culture. The other strain, after 12 months in tissue culture, caused parasitic and thermal reactions in some of the inoculated animals but when schizonts of this strain, which were in tissue culture for 21 months, were inoculated into animals, neither thermal nor parasitic reactions followed. It is not clear at what point exactly this strain became attenuated. These authors pointed out that both strains lost the ability to produce erythrocytic forms before they became attenuated

to the extent that schizonts could not be detected in the inoculated animals. In the present study 4 strains of T.annulata were studied for attenuation in tissue culture. Strain A which was studied for 1110 days in tissue culture still provoked thermal and schizontal reactions, but not erythrocytic forms, in inoculated animals. Although these reactions were not severe they were very similar to those induced by this strain after one year in tissue culture. Strain B was rendered completely attenuated after 110 days in tissue culture. Animals inoculated with the schizonts of this strain, which were in tissue culture for 110 days or more, showed neither thermal nor parasitic reactions. Strain C at the end of the present study after 240 days in tissue culture caused only slight thermal reactions. Strain D was different from the other 3 strains. It caused extremely mild reactions from the first passage after 4 days in tissue culture. This character was maintained even after 165 days in tissue culture whereas this strain, after blood passage, when used in challenge tests caused severe reactions in the control and some of the vaccinated animals. The results of the present study showed that, apparently, strains of T.annulata in tissue culture shared a common phenomenon in the loss of ability to produce erythrocytic forms of the parasite, when they were inoculated into susceptible animals. The required time before they became so attenuated as not to cause thermal or schizontal reactions in the inoculated animals varied from strain to strain.

It was achieved in Strain B in a period as short as 110 days but in Strain A such degree of attenuation did not occur even after 1110 days *in vitro* culture. The author of this thesis suggests that one of the possible roles of tissue culture in attenuation of *T.annulata* strains might be the selection of a parasite with low virulence. For explanation of this it seems necessary to introduce an hypothesis. It is already known that there are strains of *T.annulata* in nature with varying antigenicities and virulences (Neitz 1957). When ticks feed on a carrier animal they infect themselves with one or other strain. When a susceptible animal becomes infested with a number of infected ticks, possibly, many strains will be introduced into the animal's body simultaneously and, therefore, the theileriosis that occurs might be due to more than one strain. The biopsy materials that are used for isolation of *T.annulata* infected lymphoid cells might contain schizonts of more than one strain. As the subcultures are repeated dilutions of the cells in culture, it is possible that during such subcultures strains with varying virulence are selected out. On the grounds that strains of *T.annulata* with lower virulence grow better in tissue culture (Hooshmand-Rad and Hashemi-Fesharki 1968) the result might be the selection of an avirulent strain or one of low virulence.

3 - Storage of infected lymphoid cells

Low temperature storage of living material became a

more practical proposition when Polge, Smith and Parkes (1949) observed that the addition of glycerol to semen diluents protected spermatozoa during freezing. Glycerol as a cryoprotectant has been used since then for the preservation of a wide variety of cells including protozoa. There are other compounds which are also used as cryoprotectants. Dimethyl sulphoxide (DMSO), used for the first time as a cryoprotectant by Lovelock and Bishop (1959) to protect erythrocytes and spermatozoa during freezing and thawing, has emerged as an effective cryoprotectant for parasitic protozoa (Diamond 1964). According to Lovelock (1953a and 1953b) glycerol protects cells during freezing by preventing an excessive increase in the concentration of damaging salts as water in the cell suspension turns into ice. The addition of cryoprotectants, however, does not guarantee the survival of frozen protozoa. Other important factors include the rate of cooling of the parasites during freezing, the temperature of storage and the warming rate after storage (Dalgliesh 1972). Information on the storage of T.annulata infected lymphoid cells is scarce. Tsur et al. (1964) mentioned the use of 10% and 15% glycerol for the preservation of infected lymphoid cells but gave few details. In the report of these authors neither the method of freezing nor the method of thawing were clearly defined. It is not clear whether they observed any difference between the effect of 10% and 15% glycerol. Hulliger (1965) stated that infected lymphoid cells were

preserved at -79°C. in the presence of 10% glycerol. These cells were defrosted rapidly, centrifuged immediately and resuspended in culture medium. The detailed results of recovery that the above author obtained were not given. In the course of the present work a fairly comprehensive study of the preservation of T.annulata infected lymphoid cells was carried out. The length of time that infected lymphoid cells could be preserved at ambient temperature in a tropical country, with a recovery rate of 10% or more, was less than 24 hours. The storage of these cells at 4 - 8°C. prolonged the period during which 10% or more of these cells could survive to nearly 120 hours. The presence of glycerol at this temperature as well as at ambient temperature appeared to be detrimental to the cells although this effect was less pronounced at 4 - 8°C. The reason for the addition of glycerol to cell suspensions at these temperatures was to reproduce the conditions obtaining in frozen vaccine after being thawed. Storage of infected lymphoid cells at -22°C. was unsuccessful. This is in agreement with the current theory that the critical temperature range for vulnerability of living cells is from just below freezing point to about -40°C. (Lovelock 1953a and 1953b). For example, Polge and Soltys (1957) observed that in the presence of 5% glycerol only 10% of trypanosomes could be recovered after 3 days storage at -20°C. whereas 80% of the parasites could be recovered after 250 days storage at -79°C. It was found that 10%

DMSO was protecting the cells better, during freezing and thawing, than glycerol at 7.5, 10 or 15%. The optimum strength of glycerol for protection of infected lymphoid cells during freezing and thawing was 7.5%. At this level glycerol was almost as effective as 10% DMSO. For the reasons explained in the conclusions on Chapter 3, glycerol was used in the preservation of vaccine and DMSO for the preservation of stabilates. Cooling by an uncontrolled slow method was as effective as by the controlled slow method (1°C./minute). This was extremely helpful in freezing the suspensions of infected lymphoid cells to be used as vaccine. Small volumes of suspensions of infected lymphoid cells are easy to freeze by slow controlled cooling but the freezing of suspensions in a few hundred vials by this method, unless an automatic freezing bath is available, is virtually impracticable. A very critical factor in recovery of infected lymphoid cells, by means of tissue culture, after freezing and thawing was the method of elution of the cryoprotectants. The rate of recovery increased considerably when elution took place, slowly. The recovery rate of cells which had been frozen in the presence of 10% or 15% glycerol was very low if elution was rapid. It is postulated that when frozen and thawed cells are inoculated into animals subcutaneously or intramuscularly elution takes place slowly.

4 - Preparation of tissue culture vaccine

The method of preparation of T.annulata blood vaccine

was described by Sergent *et al.* (1945) and this became the method in general use. Pipano and Tsur (1966) who attempted to use infected lymphoid cells from tissue culture as an immunizing agent in cattle, stated that these cells were obtained from the supernatant fluid of their cultures but gave no specific details. In the present work a detailed description of the method of preparation is given. A vaccine, so produced, if it is preserved at a fairly regular low temperature of approximately -70°C. will keep stable for a considerable length of time. It has the advantage that it can be tested for effectiveness as well as for the presence of extraneous pathogenic agents, prior to its administration in the field.

5 - Comparison of fresh and frozen tissue culture vaccine

Blood vaccine of *T.annulata* had to be used freshly and, therefore, it had all the disadvantages of such a type of vaccine. The successful recovery of schizonts in lymphoid cells after freezing and thawing suggested that frozen infected lymphoid cells could be used as an immunizing agent. Tsur *et al.* (1964) pointed out that frozen infected lymphoid cells produced schizonts when they were inoculated into susceptible animals. The author of this thesis was confident that frozen culture would provoke reactions but in order to compare the reactions resulting from vaccination with fresh culture and those caused by frozen culture an experiment was carried out. It was

shown that the reactions induced by both types of vaccine were similar and for the reasons mentioned in the discussion on Chapter 5 the minor differences were considered to be negligible. The author accepted that the immunity engendered by either vaccine would be of the same order and, therefore, chose to use the frozen material.

6 - Reaction of animals to doses of different size

At the time when blood vaccine against tropical theileriosis was in use the dose had to be determined from year to year (Sergent et al. 1945). It ranged from 5 to 10 ml. of infected blood. Tsur et al. (1964) reported that the number of schizonts in the immunizing inocula they used ranged from 5×10^5 to 10×10^6. Pipano and Tsur (1966) used 10^6 to 1.5×10^6 schizonts for immunization of animals. These authors neither described the degree of the reaction of animals to various doses nor recommended a specific number of schizonts in the inoculum. The difference in the numbers of schizonts in the frozen and fresh culture also was obscure. The determination of the size of dose of vaccine, for any particular strain, which would produce a reaction and immunity is of significant importance. In the present work reactions of animals to various doses of frozen and fresh tissue culture vaccine were recorded. It was observed that 1.5×10^6 schizonts of fresh culture produced a mild reaction within 12 days of the time of inoculation. A similar number of schizonts,

but after freezing and thawing, produced a reaction of the same intensity but with a longer period of incubation which was undesirable. When 3×10^6 schizonts in the frozen culture were inoculated the incubation period and severity of the reaction were similar to those produced when 1.5×10^6 fresh culture schizonts were used. This number of schizonts was accepted as satisfactory for use in the immunizing inocula as concluded in the discussion to Chapter 5.

7 - The reactions of animals relative to the route of inoculation

Sergent et al. (1945) stated that the route of inoculation of infected blood affected the severity of the resultant reaction in the inoculated animals. They considered that the intravenous inoculation of infected blood caused a severe reaction but that intraperitoneal inoculation of the same material produced a less severe infection than was caused by inoculation by any other routes. They recommended that blood vaccine should be administered subcutaneously. Pipano and Tsur (1966) used the subcutaneous route for the vaccination of their experimental animals with a tissue culture vaccine. In the present work intravenous, intraperitoneal and intradermal routes of inoculation were not considered to be of practical value and, therefore, were not used. However, in one experiment when 150×10^6 schizonts were administered subcutaneously to 2 calves and intravenously to 2 calves,

one of the calves that received schizonts intravenously reacted severely and died of theileriosis. This added weight to the contraindication of the intravenous route for inoculation of vaccine. On the other hand, the subcutaneous and intramuscular routes which are more practicable routes of the administration of vaccine in the field were used and the results were compared. The subcutaneous route had the advantage that it caused noticeable enlargement of the local lymph nodes. This was discernible from the fourth or fifth day after vaccination. The use of the intramuscular route of inoculation with tissue culture vaccine lacked this advantage. This route also had the disadvantage that there was the possibility of fatal generalization. There was no indication of such a risk even when 150×10^6 schizonts were inoculated subcutaneously. For the reasons indicated in the discussion on Chapter 5, the subcutaneous route was chosen for the definitive tests.

8 - The reactions of animals relative to age

According to Sergent et al. (1945) bovine animals of both sexes are equally susceptible to natural tropical theileriosis except that milking and pregnant cows show a greater susceptibility. Adler and Ellenbogen (1934) observed that in naturally infected calves of imported races the mortality was much smaller and the disease ran a milder course than in adults. The age of the host at the first infection affects the response to T.parva

(Barnett 1968). This was shown for a strain of zebu cattle having an innate resistance to T.parva, but the experiment was conducted on progeny which had been reared under tick controlled conditions. No such difference of susceptibility so far has been described for response to inoculation with cultures of T.annulata. Pipano and Tsur (1966) used calves and heifers in their experiment on T.annulata tissue culture vaccine. There is no indication that they observed any difference between the susceptibility of calves and heifers. This was, possibly, due to the fact that they used a highly attenuated strain for vaccination of these animals. The author of this thesis (unpublished work) observed greater susceptibility of pregnant and milking cows to Strain A when it was used for vaccination of animals in Iran. In the present work the reactions of animals of different ages were compared and it was found that animals 16 to 18 months old show a much greater reaction than animals 7 to 9 months old. The severity of the reaction was manifested in the longer duration of parasitosis and pyrexia. In such a case, if a highly attenuated vaccine cannot produce sufficient immunity in adult cattle, the alternative would be a double vaccination programme, the first vaccination to be with a highly attenuated vaccine and followed, in a few months time, with another vaccination using a less attenuated strain.

9 - Duration of immunity after vaccination

The immunity following a recovery from natural

T.annulata infection protects indigenous as well as exotic cattle against reinfections (Sergent et al. 1945). The same authors state that the immunity following an artificial infection by blood vaccine can protect most of the vaccinated animals against field exposure to natural infection. This view is not shared by all the investigators. Adler and Ellenbogen (1936) found that the Algerian strains "Kouba" and "Brunett" did not confer satisfactory immunity to exposure in the field, although they reduced the mortality. Pipano and Tsur (1966) found that cattle inoculated with tissue culture vaccine of T.annulata were immune to subsequent challenge by artificial passage of infected blood. They did not state the nature of the challenge strain they used. From the data in their paper it is assumed that a virulent homologous strain was used. They suggested that further study should be carried out to investigate the duration of the immunity engendered by tissue culture vaccine. In the work presented in this thesis it has been shown that the immunogenic properties of a strain may differ from those of other strains. (Chapter 4 comparison of the challenge of cattle vaccinated with Strains A and B). Challenge of vaccinated calves 4 months after vaccination revealed a significantly high degree of immunity, despite the fact that the immunizing and the challenge strains were from 2 different countries. Thermal and parasitic reactions of the vaccinated animal could be detected only by close observations. When the results were statistically analysed it was shown that a

significant difference existed between the reactions of the vaccinated and those of the control animals. In the present work study of the duration of immunity was extended to 12 months which was longer than any previous experimental challenge test carried out, following vaccination with tissue culture vaccine. The reactions of animals challenged 12 months after vaccination were somewhat more severe than those which were challenged 4 months after vaccination. This indicated that the immunity had commenced to wane but it was still strong enough to protect the animals by reducing the severity of the thermal and parasitic reactions. This was supported by statistical analysis of the data obtained from the challenge test 12 months after vaccination which indicated less severe reactions in the vaccinated animals than in the control animals. It appears that the immunity engendered by the inoculation of tissue culture vaccine is similar to that following vaccination with blood vaccine. Sergent et al. (1945) were of the opinion that vaccination should be repeated every year or that animals should be exposed to natural infection in the field to boost their immunity. The author of this thesis suggests that tissue culture vaccine should be used 3 months before the season when ticks become abundant, the animals being exposed to a moderate tick infestation. Subsequently by spraying animals with acaricide every fortnight or every month, the burden of tick infestation can be decreased. Vaccination at this time has the advantage that the reaction occurs in

the cooler months and so the strain on the animals is reduced.

Although the work presented here has helped, to some extent, to elucidate some of the practical aspects of the preparation and the use of tissue culture vaccine the information available is far from complete. One of the subjects requiring further investigation, for example, is the nature of the immunity engendered by tissue culture vaccine. The current concept of immunity to T.annulata is premunition or coinfection. This means that the immunity persists as long as the parasite remains in the body. The vaccine strain does not produce erythrocytic forms and, therefore, it must be assumed that the immunity is related to the macroschizonts. This immunity should be considered as a sterile immunity because, as yet, the site of persistance of macroschizonts is not known with certainty and this is a matter for future investigation. Further, the connection, if any exists, between the severity and type of response to vaccination and the degree of immunity engendered should provide an interesting field of research. Reference to the discussion on Chapter 6 indicates that the severity of the response to vaccination had no bearing on the immunity to challenge. Barnett (1963) expressed a similar opinion when discussing a second infection with T.parva in that there appeared to be no correlation between the severity of the first infection and the degree of immunity engendered. From the study carried out on the immunogenesis of T.annulata tissue

culture vaccine the points which have emerged can be summed up as follows:

1 - The immunogenic properties of T.annulata vary from strain to strain.

2 - Tissue culture vaccine can produce a marked immunity, the nature of which is not clear yet. This immunity appears to wane but persists in some degree up to one year.

3 - Vaccination should be applied in such a way that the immunity is enhanced, either artificially or by natural exposure, before it diminishes beyond the point when it can still prevent a fatal episode or even a serious clinical manifestation. It is suggested that vaccination should be carried out a few months before the seasonal flush of ticks, the animals thus being exposed soon after vaccinal immunity has developed and experiencing an enhancement of immunity. If adult animals are to be vaccinated, due consideration should be given to the fact that susceptible adults are more prone to severe reactions than are young animals. It is, therefore, advisable that discrimination should be used in the choice of strain for vaccine preparation and the degree of attenuation.

4 - The experience gained in this work indicates that tissue culture vaccine against tropical theileriosis might prove to be a valuable weapon in the control of this disease. It is important, however, that

suitable strains of parasite should be adapted for the preparation of vaccine in tissue culture having regard to the prevalent local strains in the area affected, their adaptability to tissue culture and their capacity for suitable attenuation.

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APPENDIX TABLES

Appendix Table 1.

Daily body temperatures of 2 groups of calves vaccinated with fresh and frozen culture.

Animal No.	Type of Vaccine	Days after vaccination																				
		5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1178	Fresh culture	102·3	102·3	102·4	102·3	102·4	102·8	103·8	104·4	104·0	103·6	102·4	102·3	101·4	102·5	101·8	102·0	101·8	102·0	102	102·2	102·1
1159	"	102·4	102·4	101·8	101·8	102·0	104·8	103·0	103·8	104·5	105·0	103·0	101·8	100·8	102·0	101·4	101·6	101·8	101·8	102·2	101·8	102·2
1186	"	102·4	102·0	102·4	102·4	102·3	103·0	104·0	104·4	103·0	102·4	102·8	102·8	102·9	101·4	102·2	101·8	101·4	102·1	102·1	102·2	101·8
1176	"	102·5	103·2	102·2	102·2	102·4	102·3	102·8	104·6	104·0	103·6	103·0	103·2	103·3	102·0	102·2	102·2	102·2	101·8	102·2	101·8	101·8
1182	"	102·8	102·8	103·2	102·6	102·6	102·0	102·0	102·2	103·4	104·2	103·6	103·0	102·0	102·4	102·2	102·0	101·8	102·2	102·4	101·8	101·8
1165	Frozen culture	103·0	102·2	102·2	101·4	102·0	103·0	101·6	102·7	103·4	104·0	104·8	103·4	103·0	102·0	102·2	101·9	101·7	101·4	101·6	102·2	102·0
1158	"	102·9	102·8	102·8	103·0	102·3	102·3	102·4	102·4	104·4	104·6	105·0	103·6	102·4	102·0	101·8	102·0	102·1	102·2	102·3	101·8	101·0
1164	"	102·4	102·8	102·3	102·0	102·6	102·4	102·8	103·0	103·2	103·9	103·9	102·6	102·0	102·2	101·8	101·8	102·0	101·8	101·0	101·5	101·4
1135	"	101·6	102·1	102·2	101·0	101·6	102·4	101·6	102·0	102·4	104·3	103·0	102·4	101·6	102·2	101·8	102·2	101·8	102·2	102·2	102·6	102·4
1153	"	102·8	102·8	102·0	101·6	102·2	101·6	102·4	103·2	104·4	102·4	101·8	102·0	101·8	101·9	101·6	101·3	102·2	102·0	101·8	102·6	102·8

Appendix Table 2.

Daily body temperatures of calves vaccinated with various doses of fresh or frozen cultures.

Animal No.	Type of Vaccine	Dose schizonts	Days after vaccination																				
			5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1082	Frozen culture	$1·5x10^6$	101·6	101·6	101·7	102·1	101·8	101·8	101·8	101·8	102·1	102·2	102·2	103·8	103·4	102·4	102·2	102·1	102·2	102·1	102·1	102·2	102·3
1078	"	"	102·0	102·2	102·3	101·8	102·2	102·2	102·4	101·4	102·0	102·8	101·6	102·0	102·0	103·2	103·4	103·5	105·2	106·2	105·6	102·4	101·6
1170	"	"	101·8	102·0	101·8	102·0	101·8	102·0	102·2	102·6	101·8	101·9	101·8	102·6	103·9	104·0	102·8	102·2	101·8	102·2	102·2	102·0	102·2
1154	"	"	102·1	102·0	101·8	102·2	101·8	102·2	102·4	101·8	102·3	102·1	102·0	103·2	105·1	105·1	104·4	103·4	101·4	101·9	101·8	101·8	102·4
1184	"	$3x10^6$	100·6	100·8	100·8	101·0	101·2	101·8	101·4	103·2	104·2	104·4	103·0	101·6	101·4	101·2	101·1	101·0	100·6	100·8	100·6	100·8	100·8
1144	"	"	101·2	101·2	101·6	101·6	101·4	101·4	101·6	103·2	103·9	104·2	103·0	101·0	101·4	101·3	101·0	101·3	101·3	101·2	100·8	101·0	101·2
1024	"	"	101·2	100·8	101·2	101·4	101·5	101·4	101·2	102·0	103·4	104·8	103·2	101·6	101·0	101·2	101·1	101·0	101·2	101·0	101·2	101·1	101·0
1160	"	"	102·2	102·2	102·2	102·1	102·2	102·0	102·4	103·3	104·0	103·4	102·0	102·2	102·2	102·4	102·3	102·2	101·8	102·2	102·5	102·2	102·2
1114	"	$6x10^6$	101·2	101·2	101·4	101·6	102·0	103·9	103·0	102·4	102·8	102·0	102·4	102·0	101·6	101·6	101·6	101·8	101·6	102·0	101·6	101·8	101·8
1152	"	"	101·4	101·6	101·2	101·6	101·4	101·4	103·3	104·2	104·0	102·6	101·6	101·7	102·3	102·4	102·3	101·8	101·8	102·1	102·1	101·8	102·1
1168	"	"	101·8	102·2	102·2	102·3	101·9	102·5	103·3	103·0	102·3	102·1	102·2	102·4	102·0	102·4	102·3	102·4	102·5	102·6	102·4	102·6	102·6
1046	"	"	101·8	101·8	101·8	102·2	102·8	104·0	105·6	104·4	103·0	102·0	102·2	102·2	102·0	102·3	102·1	102·0	102·2	102·2	101·8	102·0	101·8
1252	Fresh culture	$1·5x10^6$	102·4	102·0	102·4	102·0	102·0	102·0	102·5	102·8	103·6	104·2	103·4	103·8	102·1	101·8	101·6	102·0	102·5	102·9	101·6	102·4	102·0
1260	"	"	103·0	102·0	102·8	103·0	102·5	102·0	102·4	102·6	102·8	103·6	104·6	103·6	103·2	102·0	101·8	101·8	102·4	102·5	101·9	102·4	101·8
1240	"	$15x10^6$	102·0	102·6	102·8	102·9	102·8	104·0	105·4	105·8	105·0	102·8	102·6	102·8	103·6	102·3	102·8	101·8	101·9	102·0	102·6	101·5	102·4
1248	"	"	102·6	103·2	102·2	102·0	102·6	104·2	104·4	105·4	105·4	104·8	103·1	102·1	102·6	102·8	102·6	102·4	102·0	103·0	102·1	101·9	102·2
1227	"	$150x10^6$	102·8	102·6	102·6	104·0	105·0	106·0	106·7	105·8	102·4	102·4	103·4	102·2	102·6	102·8	102·4	102·6	102·0	101·8	102·2	101·4	102·0
1229	"	"	102·0	102·6	102·9	104·4	105·2	106·0	106·6	106·0	103·0	102·5	102·2	102·4	102·6	102·4	102·2	102·0	102·1	102·4	102·8	102·0	102·4
1264	"	"	102·6	102·8	102·8	104·2	106·4	106·4	106·4	106·0	106·6	105·6	106·8	106·3									
1253	"	"	100·6	102·0	102·0	103·8	104·2	105·4	106·2	106·2	102·0	101·2	101·4	102·2	103·4	102·8	101·8	101·0	102·2	102·4	102·6	101·9	101·0

Appendix Table 3.

Daily body temperatures of calves which were vaccinated subcutaneously or intramuscularly.

Animal No.	Number of schizonts	Route of inoculation	Days after vaccination										
			10	11	12	13	14	15	16	17	18	19	20
1345	$3x10^6$	s/c	102·2	102·4	102·6	103·0	104·8	104·4	101·2	102·8	101·2	102·2	102·0
1341	"	"	101·6	101·8	101·8	102·0	103·3	104·6	104·8	101·4	101·1	102·0	101·8
1378	"	"	102·0	102·4	101·4	102·8	104·0	104·8	105·6	102·0	101·6	102·2	101·6
1349	"	"	102·4	102·4	103·2	103·8	104·8	104·6	103·8	102·1	101·2	101·6	102·4
1374	"	"	102·4	101·6	101·8	101·2	103·0	104·8	104·2	101·2	100·0	101·8	102·0
1351	"	"	102·0	102·0	101·8	102·6	103·8	104·6	104·5	102·0	101·2	101·8	101·6
1234	"	"	101·5	101·4	101·4	101·6	103·0	104·2	101·4	101·2	101·8	101·3	102·0
1284	"	"	102·4	102·4	102·0	103·6	104·0	104·0	102·0	102·1	101·8	101·2	101·6
1366	"	i/m	102·2	104·2	105·0	104·0	104·0	102·2	102·0	101·6	101·0	101·2	102·0
1353	"	"	102·0	102·0	102·0	103·4	104·4	103·8	101·4	101·2	101·3	102·0	101·8
1368	"	"	101·6	102·0	102·8	103·2	104·4	105·0	101·6	102·0	100·0	102·0	101·6
1372	"	"	102·0	104·6	103·8	103·6	102·2	101·8	102·2	102·6	101·4	102·2	102·6
1360	"	"	102·4	102·0	102·4	104·2	104·2	104·8	102·0	101·6	101·8	101·8	102·0
1335	"	"	101·0	102·2	102·2	103·6	105·4	104·6	101·8	101·6	101·2	102·0	102·2
1385	"	"	102·4	102·6	104·0	104·2	103·8	101·4	101·0	101·8	101·4	101·8	101·6
1339	"	"	101·2	101·0	101·2	103·0	104·2	103·6	100·1	100·2	101·2	102·0	101·4
1355	$1·5x10^6$	"	101·0	101·0	101·4	101·6	101·6	102·0	106·0	104·2	101·6	102·0	101·8
1337	"	"	103·4	102·0	101·8	102·6	106·8	107·2	106·0	105·0	102·4	102·1	101·6
1318	"	"	101·8	102·0	102·0	102·4	106·0	104·7	103·5	104·5	102·4	102·0	102·0
1286	"	"	102·0	102·4	102·2	102·0	102·8	102·8	106·0	104·6	102·0	101·8	101·6
1343	"	"	101·2	102·4	102·4	102·8	105·4	104·0	103·0	103·6	102·2	102·0	102·0
1356	"	"	102·0	102·8	102·4	102·8	102·4	103·4	101·8	102·3	101·2	101·6	102·0
100	"	"	101·8	102·0	102·4	102·4	101·6	102·0	102·4	104·0	102·8	102·0	101·8
1354	"	"	102·0	102·2	102·0	102·0	103·0	103·4	101·8	102·8	101·4	102·0	102·0

Appendix Table 4.

Daily body temperatures of 30 vaccinated bovine animals of different ages.

Animal No.	Days after vaccination										
	10	11	12	13	14	15	16	17	18	19	20
739	102·0	102·0	103·8	104·2	102·8	103·8	101·8	102·6	102·2	101·8	102·4
763	102·8	102·8	102·0	102·8	105·4	105·8	105·0	103·4	102·0	101·8	102·0
755	102·2	102·4	103·4	102·4	102·7	104·0	101·8	102·4	102·0	101·6	101·8
767	102·4	102·4	103·8	105·3	104·2	102·8	102·0	101·8	101·8	102·2	102·0
765	102·4	102·5	103·0	103·4	105·0	103·0	104·6	103·2	102·4	101·8	101·6
745	102·6	102·7	103·0	104·8	104·6	102·4	103·4	102·0	101·6	101·6	101·8
743	102·6	102·0	101·8	103·2	103·0	102·8	103·0	101·6	101·8	102·0	102·4
757	102·4	102·5	102·6	102·3	103·0	104·4	105·0	104·0	103·2	101·8	102·2
761	102·0	102·2	103·2	104·0	102·2	103·0	102·6	102·0	101·8	102·0	101·8
753	102·6	102·0	102·0	102·8	105·0	103·0	103·4	103·2	102·1	102·2	101·6
741	102·8	102·2	103·4	105·0	105·6	104·2	102·2	101·4	102·0	102·0	101·8
749	102·0	102·4	102·6	103·8	104·5	104·6	103·6	103·0	102·6	101·8	101·4
729	102·4	102·0	102·8	103·0	104·0	104·4	105·4	103·4	102·4	102·6	101·8
751	102·4	102·6	103·0	105·0	105·2	103·2	103·0	103·0	102·0	102·2	102·2
747	102·0	101·8	101·2	102·2	103·2	103·2	102·2	101·0	101·2	101·6	101·6
1092	102·4	102·8	102·3	102·4	105·6	105·2	105·4	105·4	105·0	104·0	103·2
1072	102·8	102·4	102·6	101·4	103·4	106·0	106·6	105·6	104·2	103·0	102·6
1026	102·8	102·0	102·4	102·0	103·8	102·8	102·8	103·4	103·0	102·0	102·1
1032	102·8	102·0	102·1	102·4	102·6	102·8	103·0	103·0	103·0	104·4	105·0
1070	102·2	102·0	102·2	101·4	105·2	105·0	105·4	104·8	103·6	101·8	102·0
1010	102·6	102·8	101·2	101·2	102·2	106·0	104·6	104·0	103·8	103·6	102·8
1086	103·0	102·6	102·8	104·6	105·2	105·6	106·0	103·4	103·4	103·0	102·0
1014	102·1	101·8	102·4	102·0	102·8	103·4	104·0	102·6	102·8	102·0	102·0
1102	102·6	102·2	102·4	102·6	104·6	104·6	103·8	103·2	103·4	102·5	102·6
1050	102·7	102·8	103·0	104·0	106·4	106·2	105·0	104·8	103·2	102·5	102·0
1068	102·6	102·1	102·8	103·2	103·4	105·8	105·4	105·6	105·2	104·2	103·0
1074	102·0	102·8	102·6	102·6	103·0	104·3	106·4	104·0	102·6	102·0	102·4
1004	102·6	102·0	102·4	102·0	104·2	105·2	105·2	105·6	104·6	104·0	103·0
1018	102·6	102·0	104·0	105·4	105·0	107·2	106·0	103·2	104·4	103·4	102·8
1030	102·8	102·0	104·0	104·8	105·0	105·2	104·0	102·6	102·2	103·4	102·6

Appendix Table 5.

Daily body temperatures of the vaccinated calves in the first and the second experiments Chapter 5.

Calf No.	Days after vaccination										
	10	11	12	13	14	15	16	17	18	19	20
691	101·8	101·6	102·0	102·0	102·6	104·6	105·2	103·4	102·4	102·0	101·4
705	102·3	101·8	102·0	103·4	104·0	105·2	105·0	103·4	102·4	102·0	102·0
731	102·2	104·8	105·3	105·3	104·0	102·2	102·0	102·4	102·2	102·6	101·0
1087	103·2	102·0	102·8	103·4	104·0	104·6	104·8	104·6	102·0	102·2	101·2
1091	102·0	102·0	102·6	102·6	103·0	104·4	104·8	104·6	103·8	103·2	101·8
1093	102·4	102·6	102·6	103·0	103·8	104·2	105·0	104·8	103·2	102·4	102·4
1099	102·6	102·5	102·8	103·6	104·8	104·4	104·4	103·2	102·6	103·0	103·2
1108	103·0	103·0	103·8	102·0	102·3	102·6	102·6	102·4	102·2	102·4	103·0
1112	102·4	102·2	102·8	103·2	103·4	102·6	102·4	101·8	102·0	102·2	102·6
1116	103·4	103·2	105·4	104·0	103·6	104·0	104·6	103·8	102·4	101·8	102·0
1122	102·4	102·0	102·4	102·4	103·0	103·2	103·6	104·2	103·4	102·2	102·2
1124	102·2	103·0	102·8	103·2	102·6	102·6	102·3	102·2	102·2	102·2	102·2
1128	102·0	102·4	103·0	102·0	102·4	103·8	104·4	104·6	104·8	105·4	105·0
1130	102·8	102·4	102·2	102·0	102·0	102·8	102·8	103·0	102·4	102·2	102·2
1134	102·3	102·3	102·8	102·6	102·8	102·2	102·4	103·6	102·6	102·0	102·8
1140	102·6	102·0	102·0	102·0	102·8	102·6	103·6	103·6	102·8	102·0	103·4
1146	102·6	102·6	102·0	104·2	103·8	102·4	102·4	102·8	102·8	103·0	102·4

Appendix Table 6.

Daily body temperatures of vaccinated and control animals in the first challenge (4 months after vaccination).

Calf No.	Group	Days after vaccination																				
		10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
691	vaccinated	102·9	102·2	103·0	102·5	102·5	102·8	102·6	102·0	102·8	103·0	103·2	103·0	102·6	102·0	102·1	102·4	102·2	102·0	102·0	101·9	102·0
705	"	102·0	102·4	102·0	102·0	102·0	102·2	101·8	101·8	102·4	103·0	103·2	102·4	102·3	102·2	101·8	102·2	102·2	101·8	101·8	102·0	102·4
731	"	102·0	101·8	102·4	101·8	101·8	102·6	102·9	102·5	102·8	103·1	102·2	102·2	102·1	102·0	102·4	102·4	102·4	102·0	101·9	102·6	102·0
1108	"	102·9	102·2	102·9	102·4	102·4	102·6	102·2	102·0	102·6	102·8	103·0	103·4	102·8	102·2	102·0	102·6	102·4	102·2	102·3	102·4	102·5
1116	"	101·6	102·3	102·4	101·8	101·8	102·8	102·8	102·6	103·2	102·4	102·2	102·4	102·2	102·0	101·8	102·6	102·2	102·0	101·9	101·8	101·0
1140	"	101·8	101·4	102·0	101·6	101·6	102·2	102·8	102·4	103·4	104·6	104·0	104·0	102·8	102·6	102·4	102·8	102·4	101·4	102·0	102·3	102·0
1134	"	102·4	102·2	102·0	102·0	102·0	102·4	101·9	102·4	103·0	103·2	103·3	102·8	102·5	102·0	102·0	102·2	102·0	102·0	102·0	101·8	101·0
1069	control	102·0	102·2	102·2	102·0	101·8	101·6	103·2	103·0	104·6	103·5	103·5	102·6	102·8	102·8	102·0	102·4	102·4	102·4	102·0	102·1	102·0
1121	"	102·0	101·6	101·8	101·8	103·2	102·4	101·6	101·8	102·0	102·4	103·8	104·3	106·0	107·2	106·8	107·0	105·2	104·8	103·4	102·4	102·4
1131	"	103·0	102·2	102·8	102·2	101·8	103·4	102·2	102·9	104·0	105·2	103·8	105·0	105·8	106·3	105·2	106·2	105·2	106·0	103·2	102·2	102·2

Appendix Table 7.

Daily body temperatures of vaccinated and control animals in the second challenge (12 months after vaccination).

Animal No.	Group	Days after challenge																				
		10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1087	vaccinated	101·5	102·2	102·8	102·8	102·8	102·6	102·8	103·0	103·6	103·6	102·0	102·2	103·4	102·8	102·6	102·6	102·8	102·2	102·2	102·0	101·3
1091	"	101·7	102·2	102·6	102·6	102·2	102·0	102·2	103·4	104·8	105·8	104·4	104·4	106·4	105·9	103·0	103·7	102·8	102·6	101·3	102·0	102·3
1093	"	101·9	102·4	103·0	102·8	102·9	102·8	102·4	102·6	102·4	105·8	104·2	106·2	106·0	106·2	103·0	104·0	102·4	102·8	102·9	102·6	102·3
1099	"	101·9	102·2	102·6	103·2	103·5	103·0	102·0	102·6	103·4	105·6	103·5	103·5	103·6	102·8	102·8	103·4	102·4	102·1	102·1	101·9	101·4
1146	"	101·8	102·0	101·9	102·4	102·0	100·2	101·8	102·7	104·4	104·2	103·8	103·4	103·4	101·6	102·8	102·0	100·0	102·0	102·4	102·9	102·8
1128	"	102·9	102·0	101·4	101·9	102·2	101·6	100·6	104·4	106·2	104·2	104·0	103·7	103·0	102·0	102·4	103·0	101·0	102·0	102·6	101·0	102·8
1122	"	102·2	101·8	101·8	102·4	102·8	101·5	102·2	103·6	105·0	104·0	103·0	102·0	101·6	101·8	102·8	102·8	102·2	102·0	102·8	102·4	103·0
1130	"	101·2	102·9	102·4	103·0	102·6	101·7	102·1	102·0	105·0	105·2	103·4	102·9	102·0	102·4	101·8	102·4	102·6	102·2	101·4	101·6	102·0
1112	"	102·5	102·4	101·9	102·8	102·4	101·3	102·8	103·4	104·0	104·0	104·2	103·0	103·0	102·3	101·6	102·3	101·9	101·4	102·9	101·8	101·0
1124	"	102·7	101·6	101·5	102·1	102·6	102·4	103·4	105·6	105·6	105·2	102·4	102·8	101·8	102·0	100·9	102·2	102·6	102·5	102·4	100·9	102·6
1241	control	102·8	102·4	102·2	102·2	103·0	103·0	103·6	105·6	105·6	105·3	104·0	103·8	102·8	102·0	102·9	103·2	102·4	102·4	103·2	103·6	102·8
1243	"	102·3	102·0	102·6	102·8	103·0	103·9	104·3	104·2	104·6	103·6	104·0	104·0	103·0	102·9	102·6	103·0	102·2	102·6	102·0	103·0	102·8
1245	"	102·9	102·8	102·0	101·8	103·0	103·4	103·4	104·5	105·0	104·4	104·0	103·9	103·4	102·0	102·6	102·0	102·9	103·0	103·0	102·0	102·8
1247	"	102·6	102·4	102·9	102·0	102·4	103·2	103·6	106·6	106·7	106·2	105·0	103·0	103·0	102·0	102·4	102·4	102·8	103·0	102·9	102·8	102·9
1249	"	102·8	102·2	102·2	103·0	104·0	105·0	106·2	107·8	107·8	107·0	106·0	104·4	104·0	104·0	103·9	103·0	102·9	102·0	102·8	103·0	102·2
1251	"	102·4	102·8	102·9	103·0	103·9	104·4	104·4	106·5	106·8	106·4	105·2	104·0	102·7	101·5	102·6	102·4	102·2	102·2	102·4	103·4	102·2
1109	"	102·5	102·9	105·0	106·0	106·0	106·9	107·0	107·0	107·0	106·9	105·3	105·4	104·0	103·8	103·0	102·0	102·4	101·4	102·9	102·9	103·0

Appendix Table 8.

Red cell counts, packed cell volumes and haemoglobin estimations of vaccinated animals, Experiment 1, Chapter 5.

	Animal No	Day -1	Days after vaccination 4	8	12	16	20	24	28	32
Red cells (10^6/cubic mm.)	691	7·25	6·40	5·20	6·70	5·76	5·18	6·13	6·00	5·90
	705	7·30	6·70	6·20	6·30	6·50	5·80	6·10	5·80	5·70
	731	6·22	5·64	6·20	6·30	5·65	6·00	5·90	5·90	5·74
	1108	6·41	5·93	6·96	6·52	6·12	6·66	6·10	6·54	6·45
	1116	8·35	7·57	7·43	6·85	6·53	6·79	7·21	7·17	7·42
	1134	8·41	7·86	7·54	7·67	6·75	6·59	6·84	7·48	7·53
	1140	7·25	8·37	8·29	7·47	7·28	6·23	6·41	6·92	6·15
Packed cells volume (%)	691	30	32	27	27	26	25	27	27	29
	705	30	29	29	27	27	26	28	29	29
	731	29	29	28	26	24	25	23	23	23
	1108	31	30	32	31	32	32	32	33	32
	1116	35	35	32	33	33	34	32	33	33
	1134	35	34	33	33	32	32	32	33	33
	1140	35	35	36	36	32	32	30	30	30
Haemoglobin (g/100 ml)	691	11·0	11·0	9·9	9·0	8·9	8·2	8·5	9·0	9·0
	705	11·0	10·5	10·5	9·8	9·0	8·5	9·0	9·0	9·0
	731	10·5	9·0	9·0	10·0	9·0	9·0	8·0	8·5	8·5
	1108	10·5	10·5	10·7	10·5	10·2	10·0	9·9	10·0	10·1
	1116	11·0	10·5	10·0	9·9	9·5	9·5	10·2	10·2	10·3
	1134	12·0	11·4	10·7	11·4	10·2	10·5	10·7	11·6	10·9
	1140	11·0	11·4	11·6	10·7	10·2	9·5	9·5	9·1	9·5

Appendix Table 9.

Red cell counts, packed cell volumes and haemoglobin estimations of vaccinated animals, Experiment 2, Chapter 5.

	Animal No	Day -1	Days after vaccination 4	8	12	16	20	24	28	32
Red cells (10^6/cubic mm.)	1087	7·58	7·82	8·50	8·09	7·72	7·70	7·36	8·41	8·21
	1091	8·00	7·85	7·51	7·25	9·38	8·15	8·60	8·44	8·73
	1093	6·25	6·72	6·05	8·44	7·45	8·12	8·08	8·71	7·05
	1099	7·35	7·11	6·59	7·25	8·55	7·95	7·53	7·41	7·01
	1112	7·35	7·62	6·92	6·86	6·85	6·35	6·51	7·23	7·22
	1122	6·45	6·34	5·93	6·17	5·83	5·64	5·91	6·25	6·73
	1124	9·77	9·54	10·53	9·15	8·59	8·91	9·22	9·34	8·93
	1128	8·45	8·35	8·11	7·92	7·32	7·11	7·65	7·91	8·22
	1130	6·74	7·06	7·39	6·83	7·65	7·53	6·87	6·74	7·19
	1146	8·25	7·95	8·20	7·95	7·21	7·80	8·15	8·05	8·43
Packed cells volume (%)	1087	34	34	38	37	34	34	33	34	37
	1091	38	38	36	38	38	39	36	35	35
	1093	36	35	36	33	34	35	35	35	34
	1099	35	34	35	34	35	34	35	35	34
	1112	33	33	32	30	30	30	30	31	32
	1122	31	31	30	31	30	30	30	31	32
	1124	40	39	40	38	36	38	39	39	38
	1128	36	36	36	35	35	33	32	33	35
	1130	33	34	34	32	34	34	33	32	33
	1146	36	35	36	35	34	35	36	36	36
Haemoglobin (g/100 ml)	1087	11·0	11·1	11·5	11·7	11·5	11·5	10·1	11·5	12·1
	1091	12·0	12·4	12·4	11·0	12·5	12·5	12·0	12·0	12·0
	1093	11·4	11·6	12·7	11·7	11·0	10·8	11·0	11·0	11·0
	1099	10·6	10·6	12·0	12·1	11·1	11·0	11·3	11·0	11·8
	1112	10·9	10·7	10·2	9·9	10·0	9·5	9·5	10·2	10·5
	1122	10·2	10·2	10·2	10·2	10·2	10·2	10·0	10·4	10·4
	1124	13·2	13·2	13·5	13·0	12·0	13·2	13·2	13·5	12·6
	1128	11·0	11·6	11·4	11·3	10·5	10·2	10·2	11·0	11·4
	1130	10·7	10·9	10·9	9·9	10·9	10·5	9·9	9·5	10·5
	1146	11·4	11·4	11·6	10·7	10·2	9·5	9·5	9·1	9·5

Appendix Table 10.

Leucocyte counts of the animals vaccinated in Experiment 1, Chapter 5.

Animal No.		Day -1	Days after vaccination 4	8	12	16	20	24	28	32
691	Neutrophils	1140	1470	2040	1060	860	1008	1225	1440	1120
	Eosinophils	120	209	0	159	86	144	98	96	168
	Basophils	0	0	0	0	0	0	0	0	0
	Monocytes	420	315	544	424	387	432	392	288	392
	Lymphocytes	4320	3256	4216	3657	2967	3216	3185	2976	3920
	Total	6000	5250	6800	5300	4300	4800	4900	4800	5600
705	Neutrophils	770	1197	1426	1008	1060	1040	1078	1060	1344
	Eosinophils	385	171	372	48	106	364	392	424	224
	Basophils	0	0	0	0	0	0	0	0	0
	Monocytes	605	513	496	288	742	364	98	318	56
	Lymphocytes	3740	3819	3906	3456	3456	3392	3432	3332	3498
	Total	5500	5700	6200	4800	5300	5200	4900	5300	5600
731	Neutrophils	1458	1200	1491	990	1288	1170	1472	1566	1547
	Eosinophils	324	288	426	360	138	315	320	432	238
	Basophils	0	0	0	0	0	0	0	0	0
	Monocytes	378	246	213	270	230	270	512	270	476
	Lymphocytes	3240	3072	4970	2880	2944	2745	4096	3132	3689
	Total	5400	4800	7100	4500	4600	4500	6400	5400	5950
1108	Neutrophils	2296	2025	2160	1932	1775	1728	2175	1914	1456
	Eosinophils	410	270	240	84	142	256	290	264	280
	Basophils	0	0	0	0	0	0	0	0	0
	Monocytes	328	405	480	504	426	448	435	528	336
	Lymphocytes	5166	4050	5120	5880	4757	3968	4350	3894	3528
	Total	8200	6750	8000	8400	7100	6400	7250	6600	5600
1116	Neutrophils	1836	1911	2296	2415	2310	2275	2241	1742	1534
	Eosinophils	408	147	410	525	440	546	415	402	354
	Basophils	0	0	0	0	0	0	0	0	0
	Monocytes	340	588	574	630	550	637	747	335	531
	Lymphocytes	4216	4704	4920	6930	7700	5642	4897	4221	3481
	Total	6800	7350	8200	10500	11000	9100	8300	6700	5900
1134	Neutrophils	2449	1944	1625	2160	2204	2000	2430	1512	1872
	Eosinophils	553	432	325	400	456	240	450	378	360
	Basophils	0	0	0	0	0	0	0	0	0
	Monocytes	395	504	455	560	456	720	900	504	504
	Lymphocytes	4503	4320	4095	4880	4484	5040	5220	3906	4464
	Total	7900	7200	6500	8000	7600	8000	9000	6300	7200
1140	Neutrophils	1963	1885	1809	1794	1630	1472	1800	1625	1768
	Eosinophils	604	435	603	468	489	448	432	500	272
	Basophils	0	0	0	0	0	0	0	0	0
	Monocytes	453	580	335	468	326	320	432	250	408
	Lymphocytes	4530	4350	3953	5070	5705	4160	4536	3875	4352
	Total	7550	7250	6700	7800	8150	6400	7200	6250	6800

Appendix Table 11.

Leucocyte counts of the animals vaccinated in Experiment 2, Chapter 5.

Animal No.		Day -1	Days after challenge 4	8	12	16	20	24	28	32
1087	Neutrophils	1449	1395	2010	1469	2250	1944	1769	2336	2414
	Eosinophils	1104	1240	335	226	360	432	366	292	213
	Basophils	0	0	0	0	0	0	0	0	0
	Monocytes	552	465	469	226	450	432	427	438	426
	Lymphocytes	3795	4650	3886	3729	5940	4392	3538	4234	4047
	Total	6900	7750	6700	5650	9000	7200	6100	7300	7100
1091	Neutrophils	1800	2002	1809	1668	2037	1898	1885	1876	1534
	Eosinophils	675	924	402	417	291	365	455	737	531
	Basophils	0	0	0	0	0	0	0	0	0
	Monocytes	450	385	469	278	485	511	520	335	413
	Lymphocytes	4575	4389	4020	4587	5887	4526	3640	3752	3422
	Total	7500	7700	6700	6950	8700	7300	6500	6700	5900
1093	Neutrophils	1886	1925	1664	1196	1310	2000	1300	1809	1500
	Eosinophils	492	525	256	312	440	600	150	268	360
	Basophils	0	0	0	0	0	0	0	0	0
	Monocytes	574	525	384	364	770	1000	300	335	420
	Lymphocytes	5248	5775	4096	3328	6480	6400	3250	4288	3720
	Total	8200	8750	6400	5200	9000	10000	5000	6700	6000
1099	Neutrophils	2010	1884	1690	1781	2112	2132	1755	1500	1400
	Eosinophils	67	314	390	548	384	410	195	375	350
	Basophils	0	0	0	0	0	0	0	0	0
	Monocytes	469	471	325	274	480	492	585	375	250
	Lymphocytes	4154	5181	4095	4247	5624	5166	3965	4000	3000
	Total	6700	7850	6500	6850	8600	8200	6500	6250	5000
1112	Neutrophils	1908	1988	2050	1450	1740	2136	1885	1599	1740
	Eosinophils	159	355	246	145	174	445	290	369	435
	Basophils	0	0	0	0	0	0	0	0	0
	Monocytes	636	639	738	435	696	534	580	369	435
	Lymphocytes	5247	4118	5166	5220	6090	5785	4495	3813	4640
	Total	7950	7100	8200	7250	8700	8900	7250	6150	7250
1122	Neutrophils	1859	1022	3198	1924	2030	1752	2366	2262	1602
	Eosinophils	143	73	82	222	290	292	507	261	534
	Basophils	0	0	0	0	0	0	0	0	0
	Monocytes	572	657	410	370	435	365	676	522	623
	Lymphocytes	4576	5548	4510	4884	4495	4891	4901	5655	6141
	Total	7150	7300	8200	7400	7250	7300	8450	8700	8900
1124	Neutrophils	1984	1885	2067	1620	1716	1846	1725	1456	1400
	Eosinophils	640	580	795	540	660	497	690	448	616
	Basophils	0	0	0	0	0	0	0	0	0
	Monocytes	320	435	477	405	330	426	483	336	336
	Lymphocytes	3456	4350	4611	4185	3894	4331	4002	3360	3248
	Total	6400	7250	7950	6750	6600	7100	6900	5600	5600
1128	Neutrophils	1975	1710	1305	1750	2288	2016	2262	1824	1793
	Eosinophils	237	342	145	125	143	252	156	76	163
	Basophils	0	0	0	0	0	0	0	0	0
	Monocytes	474	513	580	500	572	441	468	456	652
	Lymphocytes	5214	5985	5220	3875	4147	3591	4914	5244	5542
	Total	7900	8550	7250	6250	7150	6300	7800	7600	8150
1130	Neutrophils	2088	1625	2349	1827	1495	2059	1794	2050	2119
	Eosinophils	609	250	567	435	390	355	483	902	978
	Basophils	0	0	0	0	0	0	0	0	0
	Monocytes	522	375	486	522	455	497	483	492	489
	Lymphocytes	5481	4000	4698	5916	4160	4189	4140	4756	4564
	Total	8700	6250	8100	8700	5500	7100	6900	8200	8180
1146	Neutrophils	1518	1610	1404	1287	1450	2117	1449	1349	1968
	Eosinophils	207	230	208	117	290	219	189	284	164
	Basophils	0	0	0	0	0	0	0	0	0
	Monocytes	414	345	364	468	435	584	378	497	738
	Lymphocytes	4761	3565	3224	3978	5075	4380	4284	4970	5330
	Total	6900	5750	5200	5850	7250	7300	6300	7100	8200

Appendix Table 12.

Red cell counts, packed cell volumes and haemoglobin estimations of vaccinated and control animals in the first challenge (4 months after vaccination).

	Animal No	Group	Day -1	Weeks after challenge 1	2	3	4	5	6
	691	vaccinated	8·33	7·93	8·59	7·46	7·93	6·81	6·94
	705	"	7·32	6·93	6·85	6·81	6·69	7·21	7·42
	731	"	7·36	7·25	7·66	7·54	7·63	6·45	7·10
Red cells	1108	"	8·50	6·30	9·15	7·75	7·83	7·28	7·35
(10^6/cubic	1116	"	7·90	7·20	8·00	7·30	7·14	7·92	7·84
mm.)	1134	"	6·35	6·25	7·05	6·05	6·04	6·15	6·21
	1140	"	8·31	8·55	6·14	7·04	6·93	6·43	6·75
	1069	control	9·14	5·75	6·60	6·63	6·39	6·16	7·21
	1121	"	6·95	5·45	8·00	6·60	4·55	3·40	5·75
	1131	"	6·26	9·10	6·35	6·80	4·04	3·67	5·15
	691	vaccinated	32	32	31	30	31	30	29
	705	"	26	26	27	26	26	25	26
	731	"	30	31	29	30	29	29	29
Packed cells	1108	"	31	30	28	30	29	28	29
volume	1116	"	31	29	28	27	28	29	28
(%)	1134	"	28	29	29	26	27	26	26
	1140	"	26	26	26	29	27	26	26
	1069	control	32	32	32	28	30	29	30
	1121	"	28	26	28	27	19	15	22
	1131	"	29	26	25	27	17	19	24
	691	vaccinated	9·5	9·0	9·2	9·2	9·2	9·3	9·2
	705	"	9·2	9·3	9·4	9·2	9·0	9·0	9·0
	731	"	8·5	8·5	8·8	8·3	8·0	8·2	8·5
	1108	"	9·9	10·4	9·4	9·8	9·0	9·1	9·3
Haemoglobin	1116	"	9·2	10·1	9·5	9·2	9·0	8·9	9·0
(g/100 ml)	1134	"	9·5	9·6	9·9	9·8	9·2	9·5	9·5
	1140	"	8·8	9·0	9·9	9·2	9·0	9·0	9·0
	1069	control	9·2	9·2	9·5	8·8	9·2	9·0	9·4
	1121	"	9·4	9·2	9·9	8·9	5·7	5·3	6·6
	1131	"	9·6	8·9	9·9	9·5	5·9	5·3	7·2

Appendix Table 13.

Red cell counts, packed cell volumes and haemoglobin estimations of the vaccinated animals in the second challenge (12 months after vaccination.)

	Animal No	Day -1	Days after challenge 4	8	12	16	20	24	28	32
Red blood cells (10^6/cubic mm.)	1087	5·25	6·33	6·41	6·55	5·90	6·96	4·70	4·67	5·40
	1091	5·16	5·24	4·99	4·86	4·86	4·50	3·63	3·18	2·38
	1093	6·35	6·76	6·58	6·16	5·18	4·36	3·67	3 07	2·53
	1099	7·46	6·98	6·75	6·86	6·59	5·08	4·60	3·91	4·41
	1122	6·15	6·50	6·75	6·14	5·96	4·68	4·96	5·10	6·65
	1128	6·43	6·94	6·86	6·55	5·10	4·69	4·56	5·20	5·96
	1146	8·45	8·69	7·63	7·85	7·10	5·80	6·00	6·80	7·20
Packed cell volume (%)	1087	24	25	26	26	26	24	24	25	27
	1091	25	24	24	24	23	19	15	15	12
	1093	32	26	27	27	25	23	18	15	15
	1099	29	29	29	29	25	21	21	20	25
	1122	30	30	31	29	27	25	26	27	28
	1128	29	30	30	29	27	25	25	26	27
	1146	33	33	31	31	29	25	26	27	28
Haemoglobin (g/100 ml.)	1087	9·5	10·1	10·5	10·5	10·9	8·6	6·7	6·9	7·7
	1091	11·0	11·0	10·2	9·9	8·6	7·7	5·9	4·8	4·4
	1093	9·5	10·1	9·9	9·5	8·6	7·7	6·2	6·0	5·2
	1099	11·6	10·9	10·9	11·1	10·2	8·9	7·6	5·9	7·6
	1122	10·9	10·9	11·1	10·2	9·9	8·8	9·5	9·9	10·5
	1128	10·9	11·4	11·4	10·9	9·9	8·6	7·0	9·5	10·5
	1146	11·9	11·9	10·9	10·5	10·2	9·0	9·9	10·1	10·2

Appendix Table 14.

Red cell counts, packed cell volume and haemoglobin estimations of control animals in the second challenge. (12 months after vaccination)

	Animal No	Day -1	Days after challenge 4	8	12	16	20	24	28	32
Red blood cells (10^6/cubic mm.)	1241	9·31	7·86	7·36	8·13	7·11	6·56	6·29	6·65	5·76
	1243	7·52	6·70	7·34	6·99	6·72	5·50	4·90	4·75	3·65
	1245	7·65	7·31	6·60	6·27	7·13	4·95	6·20	3·75	3·51
	1247	8·53	8·34	8·02	8·31	8·27	6·41	5·80	4·53	4·29
	1249	8·36	7·97	8·53	8·55	8·99	8·40	6·64	5·43	4·97
	1251	8·16	8·42	7·45	7·99	8·70	6·26	5·58	5·12	5·48
	1109	7·86	7·63	6·52	5·26	4·15	3·92	3·40	3·12	2·01
Packed cell volume (%)	1241	34	31	30	32	30	27	25	26	24
	1243	34	28	27	26	24	22	22	20	18
	1245	29	25	25	24	22	20	20	19	17
	1247	31	29	29	28	29	24	23	18	17
	1249	34	32	32	32	33	30	27	22	21
	1251	33	28	29	30	26	24	24	21	20
	1109	32	31	29	27	24	23	22	18	12
Haemoglobin (g/100 ml.)	1241	12·0	11·5	11·0	11·2	9·7	9·7	9·6	8·7	7·6
	1243	10·8	10·5	10·0	9·4	8·6	7·5	7·5	6·6	5·8
	1245	10·4	9·8	9·8	7·2	7·0	6·5	6·9	6·2	5·9
	1247	11·0	11·0	11·0	9·7	8·6	8·1	8·0	6·1	5·8
	1249	12·0	11·2	10·2	11·0	11·4	9·2	9·6	7·0	6·2
	1251	12·5	12·5	12·0	11·0	7·5	8·1	8·0	7·2	5·9
	1109	11·9	11·6	10·9	7·5	7·0	6·1	5·9	5·9	4·6